BIOETHANOL PRODUCTION FROM DILUTE ACID-PRETREATED RICE AND SORGHUM BIOMASS VIA ENZYMATIC HYDROLYSIS AND FERMENTATION

BY

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DECLARATION

I, Agatha Kemunto Nyang'au duly declare that the work presented in this thesis is my original work and has not been presented for a degree or any other award in any other university or any other institution.

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DEDICATION

I dedicate this research work to Aloysious Nyang’au and Yunike Nyakerario, special and amazing parents.

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ABBREVIATIONS

ATR	Attenuated Total Reflection
ANOVA	Analysis of Variance
CO_2	Carbon dioxide
FAN	Free alpha amino-nitrogen
FTIR	Fourier- transform Infrared Spectroscopy
GHGs	Greenhouse gases
HPLC	High Performance Liquid Chromatography
HMF	Hydroxymethyl Furfural
KARLO	Kenya Agricultural Research and Livestock Organization
KEBS	Kenya Bureau of Standards
KIRDI	Kenya Industrial Research and Development Institute
NO_X	Nitrogen Oxide
RID	Refractive Index Detector
SSF	Simultaneous Saccharification and Fermentation
USA	United States of America

ABSTRACT

The world has a massive energy need, and bioethanol is a viable clean energy substitute for the fast-depleting fossil fuel supply. Large volumes of crop leftovers generated and left in the field for burning can be used in an affordable, dependable, and steady way thanks to production of bioethanol from lignocellulose biomass. However, because of the variations in lignocellulosic biomass, biochemical composition and lignin's recalcitrance, producing ethanol from it is still difficult. This study evaluated the effects of pre-treating selected agro wastes with dilute acid on ethanol yields via microbial hydrolysis and fermentation. Nerica husk (NH), Nerica straw (NS), Basmati 370 husk (BH), Basmati 370 straw (BS), sorghum from KALRO plot 8 (SP08) and Sorghum from KALRO plot 17 (SP17) were used in this study. Biomass samples were dried to a constant moisture content, milled into fine powder, and treated at 121°C for 60 min with sulphuric acid (1.2%(w/w) or 2.25%(w/w) at a solid to liquid ratio of 1:10. This was followed by enzyme hydrolysis using cellulase from *Aspergillus niger* at 5% substrate loading. High performance liquid chromatography (HPLC) with a reverse phase column and refractive index detector was used to evaluate the resultant sugars. The cellulase hydrolysed substrate solution was fermented with *Saccharomyces cerevisiae*, and the alcohol production was measured by HPLC. An analysis of the statistics was done through the analysis of variance (ANOVA), considering a 95% confidence level using SPSS software. Simple sugars including glucose, sucrose, maltose, and specific sugars such as xylose, arabinose, and mannose were detected, with glucose being the sugar that was found to be most prevalent in all samples. Glucose yields were consistently higher in samples pre-treated with 2.25%(w/w) dilute sulphuric acid than in biomass samples pre-treated with 1.2%(w/w) sulphuric acid, which yielded between 12.8%(w/v) and 26.7%(w/v) glucose across all biomass samples. In contrast, cellulase hydrolysis after 2.25%(w/w) acid pre-treatment, yielded 32.3%(w/w), 30.6%(w/w), 31.6%(w/w), 30.3%(w/w), 29.3%(w/w), and 13.4%(w/w) glucose from NH, NS, BH, BS, SP08, and SP17 samples, respectively. Biomass samples pre-treated with 2.25%w/w dilute acid and subjected to microbial fermentation produced ethanol ranging from 5.8%(v/v) to 8.9%(v/v). These findings demonstrate that 2.25%(w/w) sulphuric acid pre-treatment significantly enhances the release of fermentable sugars, particularly glucose from selected agro-wastes, supporting their potential as viable low-cost feedstocks for sustainable bioethanol production in Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Energy demand seems to be increasing across many countries around the world, owing primarily to increased population and industrialization (Sarkar *et al.,* 2012). Petroleum is currently the world's main energy source. However, since it is non-renewable and it is fast depleting, more renewable low-carbon energy sources need to be identified in order to minimize over-reliance on fossil fuels (Tse *et al*., 2021). Furthermore, fossil fuels are harmful to the environment because they generate significant amounts of greenhouse gases (GHGs), which contribute to global warming ((Sarkar *et al*., 2012); (A. Singh *et al*., 2011)). According to a study by Schenk *et al*., (2008), GHG accumulation has surpassed the dangerously high threshold of 450 ppm, revealing an obvious demand for carbon-free, admissible energy sources.

One viable solution to the world's growing energy requirement is the manufacturing of ethanol from renewable sources. First generation bioethanol is produced from starch-based materials such as corn, wheat, and sugar cane; however, the production process is still costly, rendering ethanol uncompetitive with fossil fuels (Vasić *et al*., 2021). Because these corn, wheat and sugarcane are also used as fodder and food for human, it is not advised to use them in the manufacturing of ethanol. Alternative raw material sources, such as lignocellulosic agricultural wastes, have thus been identified for cost-effective production of second generation (Sun and Cheng, 2002).

Lignocellulosic resources are available in large quantities throughout the year in many countries across the world. They include wastes from postharvest handling of fruits and cereal

crops. On many occasions, they are regarded as low-value materials, particularly in the developing countries where they are disposed of in landfills or burned in open fields. However, research has found that lignocellulosic waste materials are high in mixed sugars, which are useful in ethanol production (Sun and Cheng, 2002). These sugars are a significant component of their structural carbohydrates, which include lignin, cellulose, and hemicellulose (Williams *et al*., 2017).

There are three major phases in the biotransformation of lignocellulosic biomass into ethanol: substrate pre-treatment, enzymatic or chemical hydrolysis of the pre-treated substrates into fermentable sugars, and subsequent fermentation of sugars into ethanol (Wyman, 1994). Substrate pre-treatment appears to be the most limiting step, hence research into new pre-treatment methods capable of reducing lignin content while exposing the highest polysaccharides for hydrolysis is continuing. It is necessary to transform the biomass macroscopic and microscopic dimensions in order to accelerate and improve the saccharification of the carbohydrate fractions to monomeric sugars. So far, various approaches have been documented, including physical, physicochemical, chemical, and biological pre-treatments. Dilute acid is one of the pre-treatment procedures that has attracted a lot of attention over the years (Mosier *et al*., 2005), because, unlike other treatments, secondary reactions can be avoided during dilute acid pre-treatment (Hendriks and Zeeman, 2009). Despite its significance, there is limited data on the optimal dilute acid concentrations for specific local agrowastes, additionally the relationship between acid concentration is often generalized and underreported making it difficult to identify best acid concentration that is both effective, economical and adaptable(Deshavath *et al*., 2017). The acid pre-treatment stage can be followed by enzyme pre-treatment to expose more fermentable sugars such as glucose, hexoses and pentoses that can be subjected for fermentation. The recent study aims therefore to analyse

the proximate and carbohydrate composition of husks and straws from Basmati and Nerica rice varieties, as well as sweet sorghum stalks, and to determine the best pre-treatment method for ethanol production via microbial fermentation of sugar.

1.2 Problem statement

Energy demand has gradually increased over the previous century as a result of global population growth and industrialization. Petroleum is currently the main source of energy; but, due to fossil depletion and environmental concerns, low-cost energy from renewable sources with no or minimal environmental impact is becoming more attractive on both a domestic and industrial scale (Sun and Cheng, 2002).

Traditional energy crops like corn and sugarcane cannot produce enough bioethanol to meet global demand due to their primary roles as food and feed sources. Furthermore, while many plant material have been identified for bioethanol production, more agro-waste need to be explored, and their suitability evaluated. In addition, the complexity of lignocellulosic biomass particularly lignin recalcitrance makes it necessary to identify effective pretreatment methods that enhance fermentable sugar yields.

Agricultural waste present significant environmental, economic and health challenges. The common practice of burning or discarding crop residues leads to air pollution, greenhouse gases and wasted biomass resources which need to be effectively addressed and managed.

1.3 Justification

Bioethanol is a sustainable energy source that has no negative environmental impact and does not release greenhouse gases that contribute to global warming. It can be produced from microbial fermentation of agricultural waste, which is abundant on a global scale. Because of their primary value as food and feed, traditional energy crops like corn and sugarcane cannot be able to produce enough bioethanol to meet the demand worldwide. As a result, other

lignocellulosic substrates like agricultural waste are becoming more attractive as raw material for bioethanol manufacturing. More plant wastes have been classified for use in bioethanol production; nevertheless, more need to be identified and their appropriateness in bioethanol production evaluated. In addition, pre-treatment methods for lignocellulosic materials have been shown to have a considerable impact on yields of fermentable sugars, which in turn affect ethanol yields. Dilute acid pre-treatment is widely recognized for its balance of effectiveness, scalability, and cost-efficiency compared to other pre-treatment methods. Nonetheless, it is essential to identify the optimal acid concentration for each specific biomass type to enable data-driven selection of conditions that minimize the formation of inhibitory compounds while maximizing fermentable sugar yields, ultimately enhancing the overall cost-effectiveness of bioethanol production from agro-waste.

Rice and sorghum have short growth seasons and produce substantial amounts of wastes such as husks, straws, and stalks throughout the year. Despite their high content in fermentable sugars, these wastes are currently considered low value and disposed of by burning or landfill, resulting in green gas emissions that contribute to global warming. Thus, the current study investigated the effect of pre-treating rice husks, rice straw, and sweet sorghum stalks with dilute acids and hydrolysed by cellulases to produce fermentable sugars for use in the generation of ethanol by microbial fermentation.

1.4 Research Questions

i) Are the proximate composition and structural carbohydrates of the selected agro-wastes significantly different?

ii) Does pre-treatment of selected agro-wastes with varying acid concentrations result in significantly different fermentable sugar yields?

iii) Is the ethanol yield from dilute acid pre-treated selected agro wastes significantly different?

1.5 General Objectives

To characterize selected agro wastes and investigate effect of different dilute acid pre-treatment methods on yields of fermentable sugars and ethanol production via microbial fermentation.

1.5.1 Specific objectives

i) To determine proximate composition and structural carbohydrates of the selected agro-wastes.

ii) To evaluate appropriate dilute acid concentration pre-treatment for effective hydrolysis of selected agro wastes into fermentable sugars

iii) To evaluate bioethanol yields through microbial fermentation of selected acid pre-treated agro wastes

1.6 Study Gaps

I. Lack of characterization of local rice and sorghum biomass varieties in Kenya for bioethanol production
II. Limited understanding of optimal dilute acid concentrations that minimize degradation by-products while maximizing sugar release
III. Underexplored potential of converting local biomass varieties into fermentable sugars and ethanol

1.7 Significance of the study

I. The study will provide a first-time data on the biochemical composition of the local biomass varieties for bioethanol production

II. The study will develop an optimized concentration for dilute acid pre-treatment specific to Kenyan feedstock

III. This study will demonstrate a conversion potential for local biomass to fermentable sugars and ethanol

IV. It will support renewable energy innovation and value addition to agricultural residues

V. This study will contribute to the energy security and bioeconomy in Kenya

CHAPTER TWO

LITERATURE REVIEW

2.1 Status of Agro-wastes

Kenya generates approximately between 3-4million tonnes of waste per day, of this waste, about 70-80% of waste is organic consisting of agricultural waste, yard waste and food-waste (Dulo *et al*., 2022). UNEP reports that only 10% of generated waste is taken to designated disposal sites, this would therefore indicate that the bio-waste is a key contributor to waste management problem in Kenya (Dulo *et al*., 2022).

The raw materials for biofuels are derived from lignocellulosic agricultural and forest residues. Kenyan agriculture sector is known to be a main economic activity and contributes directly and indirectly to over 50% of GDP, the sector is expected to generate high quality and quantity of biowaste. In 1990 it was reported that there were about 2900 million tonnes of lignocellulosic waste from cereal crops,160million tonnes from pulse crops, 14million tonnes from oilseed crops and 540 million from plantation crops (Wu *et al*., 2018). National and International research centres have reported significant yield increases in many crops as years have progressed; however, farmers remain unaware and have low perception of the skills to take full advantage of more technologies on the production yields (Mussatto and Teixeira, 2010). Actual estimates of current agricultural waste arising are rare as they lack mapping because there are no systematically collected data (Dulo *et al*., 2022).

Second generation biofuel production has a potential of making good use of abandoned land, promoting rural development and improving economic conditions in developing regions globally depending on the choice of biomass and cultivation technique (Singh *et al*., 2011).

2.2 Composition of Agro-wastes

Agro-waste is a renewable organic matter that comes from raw agricultural products such as crops, fruits and vegetables (Obi *et al*., 2016). Plant biomass is naturally a lignocellulose material which comprises of cellulose the principal constituent, Hemicellulose, Lignin, moisture, cell wall protein, pigments and ash (Chen, 2014). The composition of these structures varies from one plant to the other (J. B. Sluiter *et al*., 2010), Hemicellulose, cellulose and lignin are joined by hydrogen bonds and besides the hydrogen bond there is a chemical bonding between hemicellulose and lignin.

Cellulose is a linear homopolymer composed of D- glucopyranose units linked by B-1,4 glycosidic bonds (Kumar *et al*., 2009). Cellulose polymer comprises amorphous and crystalline areas and exists in its crystalline form which renders its resistance to enzymatic hydrolysis and therefore produces low concentration of simple sugars (Legodi *et al*., 2021). Degradation of cellulose is an important reaction that can be used to produce simple sugars (J. B. Sluiter *et al*., 2010). The three primary pre-treatment techniques for cleaving the glycosidic bonds between two neighbouring glucose molecules are alkali, microbial and acid. Cellulose a polysaccharide with the richest source of glucose is found in lignocellulose biomass and waste including agricultural wastes, pulping wastes, cereal straws, threshing husks as well as food processing co-products such as brewers spent grain (Kumar *et al*., 2009).

Another important component of plant material is hemicellulose, a co-polymer made up of various ratios of saccharide molecules. Because of its sparse crystalline structure, it degrades readily. Lignin is the third main component of agro-wastes biomass, it is a complicated amorphous polyphenolic polymer that gives the plant its structural strength. Lignin has content of between 12% to 25% in agro-wastes (Ponnusamy *et al*., 2019). Each plant has a different

structure; for example, while wheat straws and leaves have a higher proportion of hemicellulose, hardwood trees have a bigger amount of cellulose. Furthermore, different components within a single plant have different ratios, depending on age, growth stage, and additional factors.

2.3 Agro-waste Biomass.

2.3.1 Sweet Sorghum

Sweet sorghum (*Sorghum bicolar* (L) Moench) is among the most important cereal crop globally and is ranked fifth after wheat, maize, rice and barley in relations to production and significance (Okeyo *et al*., 2020). Sweet sorghum is a grass whose stalks have a high sugar content, it has ability to perform relatively well under both favourable, tough and unpredictable weather conditions predominant in Sub-Saharan Africa and is best suited for ethanol production (Reddy *et al*., 2008). Sorghum is resistant to drought and can withstand hot spells, it is also capable of withstanding extended exposure to flooding (Muturi *et al*.,2013). About 25 million tonnes of sorghum are produced per annum in Africa and this translates to one-third of the world crop (FAOSTAT, 2008).

Sorghum is a crop that has lately gained importance in East Africa's industrial sector. Its relatively high biomass productivity and low input needs have made it a promising energy crop for the manufacturing of ethanol. In favourable environment, varieties of sweet sorghum can grow 14ft tall and produce 20-50tons of biomass (fresh weight) per acre (Mukabane *et al*., 2014). Sorghum grass is a multifunctional food-crop that can be grown for simultaneous production of grain for fodder/food and sweet sorghum stalks can be value added to produce products such as ethanol (Olweny *et al*., 2013). Sweet sorghum stalk range in diameter from

4.5mm to 1.5cm and can contain up to 75% juice, varying between 12 - 23% in sugar (Sarkar *et al*., 2012).

Sorghum is primarily grown to produce grain and feed for many low-income households in Kenya, it is majorly grown for home consumption. Sweet sorghum is a cereal indigenous to Kenya and is produced throughout much of the country, even in areas with low agricultural potential. The southwest and south-central counties of Kenya are home to the majority of the country's sorghum output. Sorghum grows in any location between sea level and 2,500 meters however it needs a minimum temperature of 10°C and minimal annual rainfall of 250 (Chemonics, 2010). Sweet sorghum is a widely adapted sugar crop with high potential for bioethanol production, it can yield more ethanol per unit area of land than other energy crops, especially under minimal input production (Regassa and Wortmann, 2014). When Sorghum gets to the mature stage, only the stalk and leaves that make up the straw can produce approximately7.81 tonnes of dry matter/ha in approximately 110 days. Since it can be harvested biannually, Sorghum may yield around 15.62 t/ha of biomass, which can be used to make ethanol of the second generation. Furthermore, 15.6 t/ha of panicles are generated, which are useful as direct feed or fodder. Sweet sorghum has the potential to yield up to 6,000 L/ha of ethanol, with an energy return of more than three units of energy produced for every energy unit invested (Regassa and Wortmann, 2014).

2.3.2 Rice husk and straws

Over half of the world's population relies mostly on rice (*Oryza sativa)*. Worldwide, rice production amounts to over 600 million tons, making it the most consumed cereal grain (FAOSTAT, 2008). Though it can be cultivated over vast parts of the world, its' physical requirement for growth is restricted to certain areas. Rice cultivation requires high average

temperature during the day but cooler nights. During the planting season, an abundant supply of water must be applied as needed, on a level land surface to support uniform flooding and drainage (Childs and LeBeau, 2023).

Rice straw and rice husk are waste products of rice production and great bio-resource. They are among the abundant lignocellulosic waste materials in the world (Nader and Robinson, 2010). Historically there is an increasing trend of the world rice production and the calculated world rice husk and straw output(Abbas and Ansumali, 2010). Rice straw is the vegetative portion of rice grass that is removed either during or after grain harvest. The agricultural residues are exploited as roofing, animal silage, shedding of homes, small-scale industries and domestic usage raw material but nevertheless, a large part of the straws and husk remain in the agricultural farms (J. Singh *et al*., 2018). Disposing of the rice straw and husk are some of the daily nightmares that farmers deal with after clearing the field. Rice straws may be burned or left on the fields before next seasons of ploughing (Nader and Robinson, 2010). Globally, the vegetative part of rice can possibly produce 205 billion litre of ethanol yearly, which equates to about 5% of total ethanol consumed. It is characterised by high hemicellulose and cellulose content, good polysaccharides for hydrolysis into fermentable sugars(Roberto *et al*., 2003).

The main constituents of rice straw are lignin (5–24%), cellulose (32-47%), hemicelluloses (19–27%), and ashes (18.8%). Pentose sugars make up the majority of hemicelluloses; xylose is the most significant pentose sugar, followed by arabinose and hexoses. The topmost stratum of rice hull, or rice husk, is the grain used in paddy fields. During milling, the brown rice is removed from the rice hull. Approximately 20% of the paddy weight in the mill is husk and most of the time it is therefore burned in open or dumped on waste land (Budki *et al*., 2012). Variations in how agro wastes are managed complicate the economic viability of farming.

Previously, burning the rice straw and rice husk was an acceptable disposable practice method but currently this practice is challenged over environment pollution and release of greenhouse gas emissions into the atmosphere. The energy value contained in the rice husk is usually as high as 410kcal/kg and therefore can be used in energy production (www.liwintermedia.com).

2.4 Fuel Ethanol

Ethanol is an ethyl alcohol that is excellent for running motor vehicles and stove fuel. It is made by biological fermentation of monosaccharides such as glucose, xylose and galactose using micro-organisms (Calam and Russell, 2007). When carrying out the process of fermentation, simple six carbon sugars are converted to alcohol and carbon dioxide. Pure ethanol burns more efficiently, has a higher-octane rating, can be burned with greater efficiency, is believed to produce reduced amounts of ozone precursors and is mainly beneficial with respect to low net carbon dioxide emission into the environmental air (Sarkar *et al*., 2012). Bioethanol is characterized by a high-octane rating of 113, which is significantly higher than that of conventional gasoline, which generally has an octane rating of 87 to 94**,** depending on the blend (Mařík *et al*., 2014). The octane number reflects a fuel's resistance to engine knocking or pre-ignition during combustion. A higher-octane rating allows for smoother engine performance and enables the use of higher compression ratios, which can improve engine efficiency (Nwufo *et al*., 2016). Lignocellulose can produce bioethanol that has the -potential to be a sustainable stove fuel, as well as a fuel oxygenate that can replace petrol since the energy required to produce ethanol is lower than the energy content in ethanol; the estimated total energy requirement for producing ethanol from corn grain is 560kJ MJ^{-1} of ethanol (Sun and Cheng, 2002).

Using bio-ethanol as a possibility for engine fuel has progressively grown globally due to various reasons. Domesticated generation and use of ethanol for fuel can reduce dependence on imported oil, decrease trade deficits, reduce air pollution, reduce global climate change carbon dioxide build up and generates job opportunities (Sun and Cheng, 2002). Ethanol contains 35% oxygen, which reduces particulate and NO_X (nitrogen oxides) emissions from combustion unlike gasoline which is non-oxygenated. Ethanol can be used as a pure alcohol in dedicated engine or mixed with petrol. Bioethanol's superior octane rating makes it a valuable additive in gasoline blends, enhancing the anti-knock properties and reducing reliance on harmful aromatic hydrocarbons traditionally used to boost octane (Iliev, 2021). This property, along with its renewable origin and cleaner-burning nature, positions bioethanol as a promising alternative fuel in the transition toward sustainable transportation, Moreover, ethanol is an excellent fuel for future advanced flexi-fuel hybrid vehicles (Zheng *et al*., 2009).

2.5 Ethanol status globally

USA, certain European countries and Brazil have embraced production and use of ethanol in large scales and is anticipated to be among the domineering renewable bio-fuels within transportation sector in the coming two decades. In as much as Kenya is yet to involve in the bio-fuels success, it is laying foundation for meaningful advancement in the years to come. Kenya enacted a policy Sessional Paper, No. 4 of 2004 and legislation (Laws of Kenya: Energy Act, 2012) the Energy Act, No. 12 of 2006 that favours the development of ethanol and biodiesel, and the Ministry of Energy has developed a biodiesel strategy through its National Bio-fuels Committee. Additionally, all facets of the biofuels industry are supporting the formation of a Kenya Biodiesel Association. The Kenyan Ministry of Industry, Trade and Cooperatives intends to focus on ethanol production. In Kenya, The Energy Act delegates broad authority to KEBS to determine fuel quality and blending standards for bio-fuels (Laws

of Kenya: Energy Act, 2012) the Energy Act,No 12 of 2006. Currently, the ethanol blending standards set by KEBS permit the blending of 90% petrol to 10% ethanol.

Ethanol can be produced artificially from petroleum or through microbial cellulosic conversion of lignocellulose materials. Ethanol for fuel market is produced from sugar and starch as in the case of Brazil and USA, respectively. However, these biomass materials, which have also been used for fodder and human consumption, are not sufficient in meeting the growing demand for bio-ethanol (Lavoie *et al*., 2008). The reduced greenhouse gas emissions resulting from the use of starch-based ethanol is not as high as desirable (Tan *et al*., 2008). These factors have called for the desire to explore Lignocellulosic feedstock such as agro-waste and forest waste as well as devoted crops and grasses such as hyacinth for the generation of ethanol. Lignocellulosic biomasses have the ability to grow on barely productive agricultural land and therefore, they can have a considerable impact on fuel needs without significantly comprising the land need for food crop cultivation.

Lignocellulosic biomass has three main components, lignin, hemicelluloses and cellulose, with the relative proportions of each dependent on the source of the raw material (Malherbe and Cloete, 2002). Proteins, plant oils, pectin and ash are compounds that contribute to the remaining percentage of the lignocellulosic biomass.

2.6 Ethanol production from lignocellulose biomass

The existing biochemical methods of converting biomass to bioethanol classically consists of 3 major steps; pre-treatment, saccharification and fermentation. The Pre-treatment procedure is done to improve enzyme accessibility to the cellulose while enzyme hydrolysis is the use of specific enzymes to breakdown the polysaccharide into monomeric sugars such as pentoses

and hexoses. The fermentation of the released sugars is then converted to ethanol by use of specialised micro-organisms.

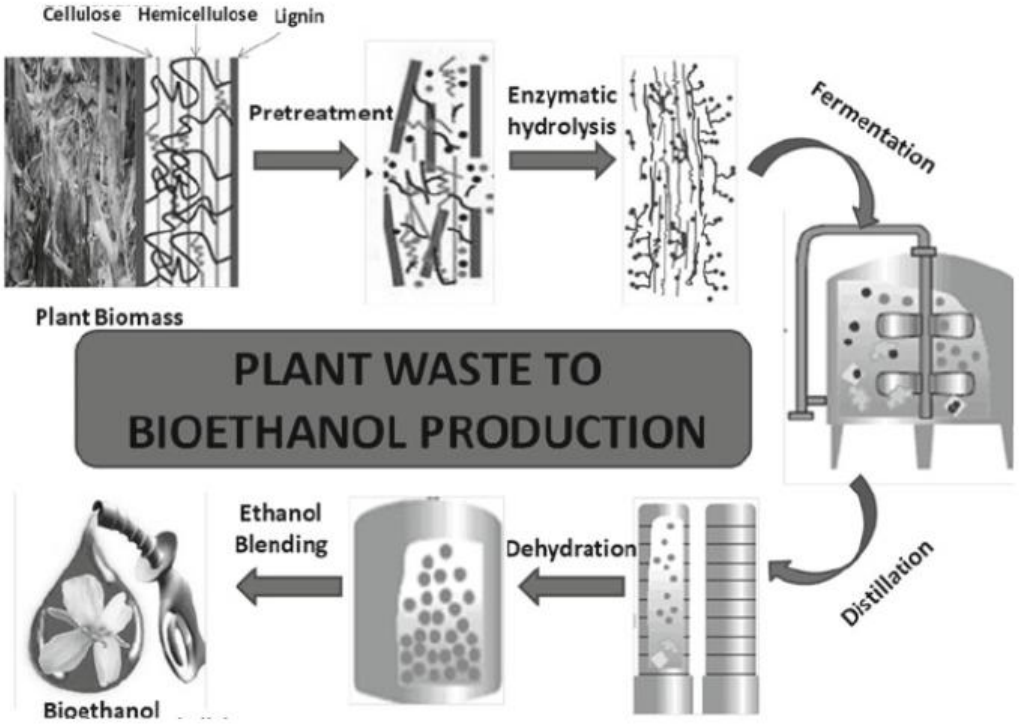

Figure 4.1: Schematic diagram of the processes of ethanol production (Hemansi *et al*., 2019).

2.6.1 Pre-treatment

Lignocellulosic material from plants is affordable and easily accessible, though the components are challenging to ferment and breakdown biologically based on the origin of the biomass. Agricultural lignocellulose contains about 50% of fermentable sugars but due to their defiant structure a prior step of pre-treatment is required. Efficient pre-treatment will reduce the size of biomass, minimise sugar loss, maximum lignin elimination with reduced formation of inhibitors(Shukla *et al*., 2023).

Pre-treatment is the process required to alter the biomass visibly and microscopically, as well as its sub microscopic structural and chemical composition to ease efficient hydrolysis of carbohydrates to simple sugars (Zheng *et al*., 2009). Lignocellulosic biomass has components

that are difficult to degrade depending on the nature of the biomass source, but carefully selected pre-treatment methods will ensure efficient conversion of the structural carbohydrates of the biomass to ethanol (Madu and Agboola, 2018).

2.6.1.1 Pre-treatment techniques

The main pre-treatment techniques are, physical pre-treatment, chemical pre-treatment, physicochemical pre-treatment and biological pre-treatment (Pendse *et al*., 2023). Understanding of various pre-treatment technologies can help select a suitable, economical and efficient combination match for a specific biomass (Rezania *et al*., 2017). Physical pre-treatment is carried out to decrease the particle size of the biomass and to increase the surface area. Milling, Freeze, microwave irradiation, extrusion is some of the techniques used in physical pre-treatment.

Chemical pre-treatment is basically the use of chemicals to transform the crystal-like structure of biomass into an unstructured form by maintaining right surrounding temperatures (Shukla *et al*., 2023). Various chemicals have various capabilities of breaking down the structure of lignocellulose biomass. Organo-solvent, alkali, acid, deep eutectic solvent, ion liquid is some of the chemical pre-treatments in use where polysaccharides, mainly the Hemicellulose are hydrolysed to monosaccharides leading to higher accessibility of cellulose to enzyme hydrolysis (Haghighi Mood *et al*., 2013). Acid pre-treatment technique can be carried out by use of concentrated or dilute acid. Industrially dilute sulphuric acid is the attractive choice of chemical because it generates lower amounts of fermentation inhibitors and studies show that low concentration acid with high temperatures gives better sugar yields (Rezania *et al*., 2017).

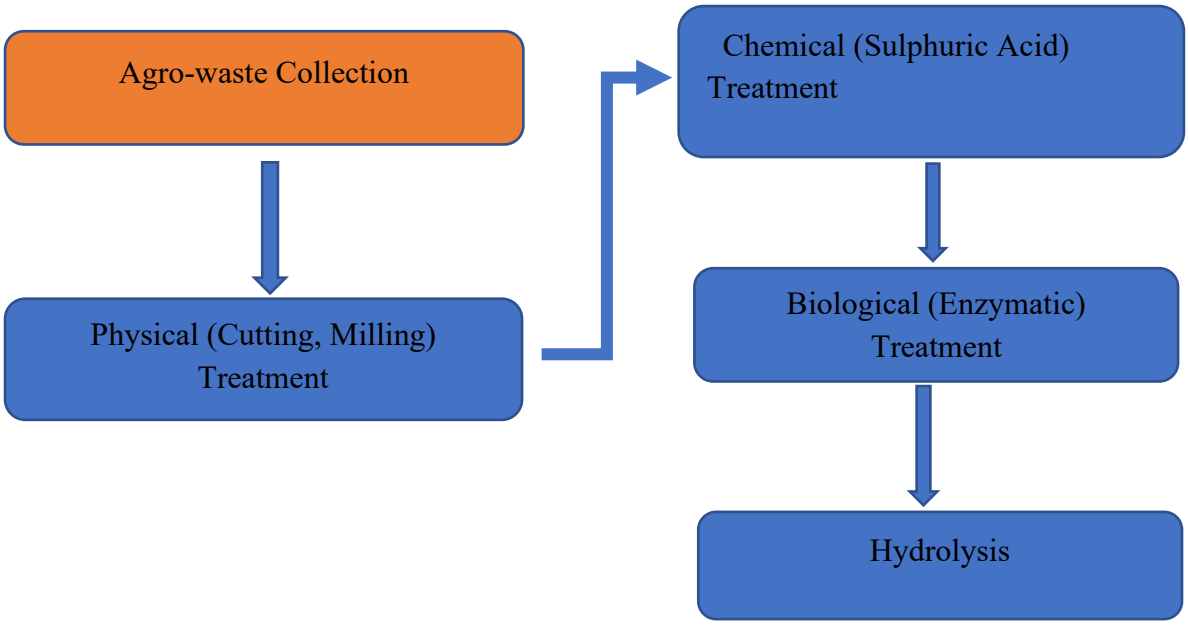

Figure 4.2: Pre-treatment process flow diagram in general (Tse *et al*., 2021).

2.6.2 Hydrolysis

Hydrolysis is the process that converts the saccharide polymers into monomeric sugars. It is achieved by mixing the pre-treated biomass with solvents to come up with a slurry which is incubated at favourable temperatures and rotated in a shaker to rupture the cell walls. Specific enzymes; cellulases which decompose polysaccharides are added during incubation (Badger, 2002) this is the enzymatic hydrolysis. Enzymatic hydrolysis by cellulases is economical because the utility cost is low compared to acid or alkaline hydrolysis because it is conducted at mild conditions of pH 4.8 and temperatures 45 - 50 °C (Sun and Cheng, 2002) and does not have a corrosion problem. Cellulases are produced chiefly by bacteria, protozoans and fungi. At optimum conditions at least three major groups of cellulases are involved in the saccharification process, the endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4.) which act on the regions of low crystallinity in the cellulose fibre, generating free chain-ends, further the breakdown of the molecule by removing cellobiose units from the free chain ends is carried out by the exoglucanase or cellobiohydrolase (CBH, 1,4-b-D-glucan

cellobiohydrolase, or EC 3.2.1.91.) finally the β-glucosidase (EC 3.2.1.21) hydrolyses cellobiose to produce glucose molecules (Ljungdahl *et al*., 1988).

2.6.3 Fermentation

Simple sugars resulting from hydrolysis process are fermented into bioethanol by ethanol producing microbes which can be found in nature or modified genetically (Zheng *et al*., 2009). The most frequently used micro-organism to produce ethanol is *Saccharomyces cerevisiae* commonly known as baker's yeast (Fan *et al*., 1980) (Badger, 2002). This is because it replicates faster, it can tolerate alcohol and has capacity for making high yields of alcohol. This yeast functions well within a pH range of 3.0 and 5.0 and a temperature range between 27 °C and 35 °C (Fan *et al*., 1980).

Saccharomyces are micro-fungi that get their energy by metabolizing organic substrates. Though glucose is mainly the main carbon source for yeast growth in the laboratory, this hexose sugar is not normally found freely in industrial fermentation. In such substrate's media, based on the promising laboratory-scale results, pilot-scale studies should be conducted to assess the technical feasibility, cost-effectiveness, and scalability of ethanol production using the best-performing biomass varieties and pre-treatment protocols identified in this study. (Walker and Stewart, 2016) states that the accessible carbon sources are maltose, sucrose, lactose and fructose. During fermentation the yeast requires correct supply of inorganic ions, especially the key metal ions. Minerals are often overlooked as key factors in yeast fermentation, even though the concentration and type of metal ions can significantly affect fermentation performance. Complex fermentation media used in industrial applications, for example molasses, whey, normally includes enough supply of inorganic ions for growth of yeast, but supplementation with additional minerals may be done occasionally.

To get pure ethanol, the product from fermentation is distilled. The best method for accomplishing this is fractional distillation, which boils the mixture of ethanol and water to achieve its mechanism of action. Ethanol with the lower boiling point (78.3 °C) compared to that of water (100 °C), will quickly be vapourised before the water is condensed and separated. Gasoline is added to the absolute ethanol obtained from the distillation to denature and dehydrate it to make it a fuel ethanol(Calam and Russell, 2007).

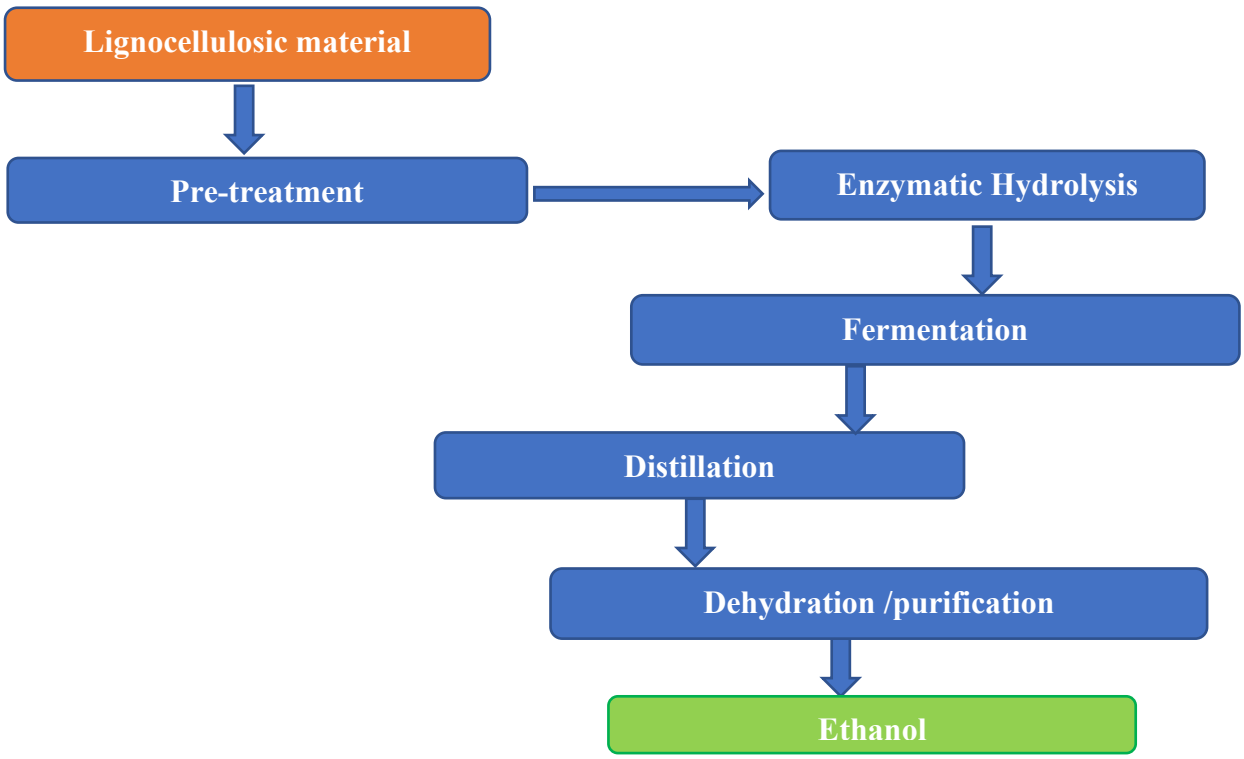

Figure 4.3: Bioethanol production flow chart. (Tran *et al*., 2020) The flow process of bioethanol production, from the raw material to purification process of ethanol.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection and preparation

Fresh rice husks and straws were taken from the Kenya Agricultural and Livestock Research Organisation (KARLO) Mwea station, while fresh sweet sorghum stalks were obtained from the KALRO Machakos station. Simple random sampling was employed, two sorghum varieties (from plots 8 and 17) were chosen, and 20 kg of each were harvested and labelled SP08 and SP17 for samples from plots 8 and 17, respectively. In addition, twenty kilograms of Nerica I and Basmati rice husks and straws were collected. Husks and straws from the Nerica 1 rice variety were labelled as NH and NS, respectively, whereas husks and straws from the Basmati 370 rice variety were labelled as BH and BS. The collected biomass samples were cut into small pieces using sickle-knife, air-dried in the sun for seven days, and milled into fine powder using a knife-mill with a 2 mm sieve. The milled samples were stored in a sealable polyethylene for further analysis

3.2 Determination of Agro-waste proximate composition

3.2.1 Moisture content analysis

The moisture content was analysed using the official methods of moisture determination and defined using equation 3.1 that was described by Sluiter *et al*., 2008). Briefly, aluminum plates were pre-dried for four hours at 105°C, cooled in a desiccator, and then their weights were ascertained. Samples (5g) were then put on previously dried plates and dried for 24 hours at 105°C in an oven then finally placed in a desiccator to cool. The sample weight was subtracted to calculate the moisture content based on weight before and after drying.

Equation 6.1: Moisture content

$$m = \frac{Wf - Wi}{W1} \times 100$$

Where W_i is the weight of the dry pan, W_f is the weight of dry pan plus sample, W1 is weight of sample as received

3.2.2 Crude fiber content

Fibre content was analysed using the standard procedure outlined in ISO 6865:2000 (International organization for standardization, 2000). 150ml of Sulfuric acid 0.15M was introduced into the powdered sample (1 g, W1) and heated for 30 min before draining the acid with a vacuum pump. The acid hydrolyzed sample was then placed in the Fibretec system (Foss company, Denmark) and rinsed three times with 10ml deionized water before being heated for 30 min with preheated 150ml of 0.23M potassium hydroxide solution. This was then washed with cold deionised water until neutral before finally washing three times with 30 mL of acetone under vacuum. Drying of the samples was then carried out at 105°C for 4 hours,cooled in a desicator and weighed (W2). The cooled sample is then incinerated in the muffle furnace at 550˚C for 2hours, cooled in a dessicator and weighed again(Wf). The crude fiber was determined by estimating the weights obtained after cooling crucibles in a dessicator in equation 3.2 below as described in ISO 6865:2000

Equation 6.2: Crude fibre Calculation

$$C\% = \frac{W2 - Wf}{W1} \times 100$$

Where C% is crude fibre (%wet) W1 is the initial mass of the sample, W2 is the weight of crucible+ residue after drying (g) and Wf is weight of crucible and residue after incineration(g).

3.2.3 Oil content

Dry samples (5 g) in thimbles (ODW) were put in soxhlet extractor and subjected to refluxing with petroleum ether for 8 h. Oil content was then estimated by substracting weight of the dry empty flask(w1) from the weight of the flask with oil(w2). Oil content was finally calculated using equation 3.3 previously described by Sluiter *et al*. (2005)

Equation 6.3: Oil content

$$0il\% = \frac{w2 - w1}{ODW} \times 100$$

3.2.4 Protein content

Crude protein content (N x 6.38) was determined according to the improved Kjedahl method (Priepke. *et al*., 1980) with slight modifications to suit the semi-automated distillation equipment. Specifically, instead of manual distillation, samples were loaded into a semi-automated Kjeldhal distillation unit, which automated the addition of sodium hydroxide and controlled steam distillation time. The use of phenolphthalein as an endpoint indicator was retained, and titration was performed manually using 0.1 M NaOH to back-titrate excess HCl

Ground sample (0.5 g) were placed in a filter paper, folded cautiously and positioned in a Kjeldhal flask. A tablet of Kjeldhal catalyst was put in a flask followed by introduction of 5 mL of concentrated H_2SO_4. A clear solution was obtained after digestion of the mixture in a fume hood for a period of 2 h. Separately, a blank was prepared by introducing a folded filter paper, a tablet of Kjeldhal catalyst and 5ml of concentrated sulphuric acid into kjedahl flask which was further digested in the fume hood alongside the samples for 2h.

Distilled water was added to the mixture after it had cooled to fill the flask to three-quarters capacity. After adding 10 mL of 40% (w/v) sodium hydroxide solution and 1mL of

phenolphthalein, the flask was connected to the distillation machine. Distillation was carried out repeatedly until no discernible reaction could be seen between a drop of distillate and Nessler's reagent in a test tube. A conical flask was used to collect the distillate containing two to three drops of methyl orange indicator and fifty milliliters of a 0.1 mol/L hydrochloric acid solution. Using 0.1 mol/L NaOH, the distillate's excess HCl solution was back titrate. The percent nitrogen was calculated as indicated by Priepke *et al*., (1980) in equation 3.4:

Equation 6.4: Protein Content

$$\%\,\text{nitrogen} = C_{HCl} \times \frac{\left(V_{HCl(s)} - V_{HCl(b)}\right) \times 14.007}{S}$$

Where: C_{HCl} = normality of hydrochloric acid; $V_{HCl(s)}$ = volume of hydrochloric used to titrate the sample in mL; $V_{HCl\,(b)}$=volume of hydrochloric used to titrate the blank in mL; S = sample weight in g %. Multiplication of % nitrogen by 6.38 factor gave as the estimated protein content of the sample.

3.2.5 Ash content

Using a pencil, 3 platinum plates were marked and approximately 5 g of each sample was put into numbered platinum plates. The samples were then charred on a hot plate in the fume cupboard till no visible smoke was present, this was then placed on a heat resistant mat and transferred to a muffle furnace heated to 550°C and heated in the furnace for 7 h. Since the sample was not yet grayish white, it was moistened with distilled water and reheated on a hot plate in the fume cupboard, then transferred to the muffle furnace for further 9 h. Samples were removed and cooled in a desiccator and weighed to determine the mass of ash (AOAC, 1990) as indicated in equation 3.5.

Equation 6.5: Ash content

$$\%Ash = \frac{Weight\ of\ Ash}{weight\ as\ received\ basis} \times 100$$

3.3 Determination of structural carbohydrate of the selected Agrowastes

The samples of the dried and finely milled agro wastes were treated with dilute sulphuric acid (final concentrations 1.2%w/w) and heated at 121°C for 60 min. The acid-treated samples were subsequently detoxified with 2M $Ca(OH)_2$ at pH 9, 50°C, and agitated for 30 min, as described by (Bjorn *et al*., (2006) The detoxified samples were then rinsed three times with distilled water before being filtered and oven drying residual solids at 70°C to a constant weight. The dried residual solids (insoluble solids) were weighed, and analysed for structural carbohydrates using an FTIR spectrometer (Bruker Alpha).

3.3.1 FT-IR Analysis

The main function of FT-IR spectroscopy is for the detection of the different functional groups present in the biomass samples (natural state and pre-treated substrates). The main results of FT-IR assays mainly deal with lignin content of the biomass. The lignin content is the focal point which is aromatic biopolymer composed of phenylpropane substituted components attached to form a giant molecule of non- consistent crystallinity and optical activity. Cellulose, Hemicellulose, and lignin must be determined as a part of an exhaustive biomass content, these polysaccharides are bound in the matrix of the biomass. Analysis of the structural carbohydrates was done using an FTIR machine. The FTIR used was the Bruker Alpha FTIR spectrometer with the diamond ATR (Attenuated Total Reflection) crystal. The crystal was cleaned before any measurement and a background done. The powder sample was ground to reduce the size of the particles to less than 5 mm in diameter. This is done because, large

particles scatter the infrared beam and cause a slope baseline of spectrum. A small amount of the sample (just adequate to cover the spatula tip) was loaded onto the ATR crystal. The scan was then done to acquire the spectrum, which was then evaluated qualitatively and quantitatively using the ATR complete library to determine the composition of the individual structural compounds.

3.4 Evaluating pre-treatment methods for efficient hydrolysis of selected agro wastes into fermentable sugar

The samples of the dried and finely milled agro wastes were treated with dilute sulphuric acid (final concentrations 1.2%(w/w) or 2.25%(w/w)) and heated at 121^{o}C for 60 min. The acid-treated samples were subsequently detoxified with 2M Ca(OH_2).

3.5 Detoxification

During Dilute acid Pre-treatment, toxic substances that inhibit yeast activity are produced, Hydroxymethylfurfural (Hmf) and furfural. The toxic substances need to be reduced or eliminated before fermentation. The process that removes the toxic substances is called detoxification, by over liming with 2M calcium hydroxide (2M Ca(OH_2) at pH 9, at 50°C for 30minutes in an incubated shaker with a 150rpm as described by Bjorn *et al*., (2006). This process intends to reduce the toxic substances. Following detoxification, the samples were cleaned thrice with distilled water, and any leftover particulates were dried in an oven at 70 degrees Celsius until their weight remained constant. For additional analysis, the dehydrated samples were kept in sealed plastic containers and stored.

3.6 Enzymatic saccharification

A mixture of substrate solution of pre-treated and detoxified samples was mixed with cellulase enzyme solution prepared using 50mM acetate buffer at 5.5 PH. The reaction mixtures were incubated at 50ºC for 72 h while being shaken at 250 rpm. The hydrolysates were centrifuged at 20ºC, at 9000 rpm for 10 min at 20ºC. Supernatants were placed in 250 mL flasks for analysis.

3.6.1 Determination of fermentable sugars

Carbohydrate content (the six carbon sugars: glucose, sucrose, maltose mannose; five carbon sugars: Xylose, Arabinose, Ribose; degradative products: HMF and Furfural) of the supernatants was determined using HPLC. The sample filtrate was collected and injected into the HPLC flow of eluent for analysis. HPLC is an extremely versatile system that separates components of a liquid mixture using the basic principle of column chromatography based on their different interactions with a stationary phase and then identified and quantified by spectroscopy. The concentration of glucose, xylose and arabinose in sample were determined by HPLC coupled with a reverse phase detector. The sugar standards were 0.01, 0.02, 0.1, 0.2, 1, 2, 10 and 20mg/ml. The column used was reverse phase column meant for organic acids and monosaccharide analysis. The mobile phase was 0.005N H_2SO_4 at a flow rate of 0.5ml/min.

3.7 Evaluation of ethanol production potential from selected agro-waste biomass

The filtrate from acid pretreated and enzyme hydrolyzed samples were prepared for distillation using the method described by (Izah and Ohimain (2015) Briefly, *Saccharomyces cerevisiae* yeast (0.1g) was added aseptically to the filtrate (100 mL) and the pH was adjusted to 4.8. The reaction mixtures were incubated at 30ºC for 168 h and agitated at 150 rpm. Resulting CO_2 was collected over water. After setting up the distillation apparatus,10ml of fermented broth were

poured into a round bottom flask and positioned on top of a heating mantle that was connected to a distillation column that was submerged in running tap water. Another flask was fastened to the other end of the distillation column to collect the distillate, and the temperature of the distillation adjusted to 78ºC. HPLC was used to calculate the ethanol production after every 4h distillation.

3.8 Data analysis

Basic statistics methods were used for data analysis. This included the mean, standard deviation, Analysis of variance (ANOVA) of the samples of sorghum, rice straws and rice husk. Turkey Post hoc test was used to determine the existence of significant difference among the means of the three groups of the selected agro-waste samples. SPSS 26 was the software which was used (www.statsoft.com/textbook/stbasic).

CHAPTER FOUR

RESULTS

4.1 General Overview

The chapter is divided into sections each covering a specific study objective. The tables and figures show results obtained for the rice and sorghum biomass treatment. The biomass characteristics, biomass pre-treatment, biomass hydrolysis and ethanol produced from fermentation were calculated. The results were averaged and expressed as means ±SEM. All the results are presented per each sorghum and Rice Varieties. The results are discussed based on samples pre-treated with 2.25% (w/w) and 1.2% (w/w) dilute sulphuric acid.

4.2 Nutritive value and carbohydrate profile of the selected agro-waste biomass

4.2.1 Proximate Analysis

4.2.1.1 Moisture content

The values of moisture were significantly different across all the biomass varieties. Sorghum varieties had the highest moisture content of 9.0% for SP8 and 8.27% for SP17. Basmati husk variety had the lowest moisture content with 4.37%. Most of the varieties had moisture content ranging between 6.3% and 8.3% as demonstrated in table 4.1.

4.2.1.2 Protein content

Comparison between the different varieties on the protein level as shown in table 4.1, showed a significant difference in all the varieties. Nerica Rice Husk variety had the highest level with 7.44% while Nerica rice straws variety had the lowest protein content with 3.46%. Sorghum varieties had the second and third highest protein content among the biomasses with 6.85% and 5.99% respectively. The Basmati rice variety had protein content ranging between 4% and 5.4% respectively.

4.2.1.3 Oil content

The oil content values of the feedstock varieties as shown in table 4.1 were significantly different across board with content levels below 1.3%. Nerica rice straw variety had the highest oil content of 1.21%.

4.2.1.4 Ash content

One-way analysis for the mean ash content in each variety across the biomasses indicated high levels of ash content in the Rice varieties as compared to the sorghum varieties. Nerica rice straws and Nerica rice husk had the highest ash content of 20.22% and 19.18% respectively as depicted in table 4.1.

4.2.1.5 Fiber content

The fiber content of the Nerica rice straws variety and the sorghum cultivars did not significantly differ from one another. Table 4.1 below shows that SP08, SP17 and NS had fiber content of about 34% which was the highest fiber content level among the biomass varieties.

Table 7.1: Nutritive value results of the selected sorghum and rice varieties in Percentages

BIOMASS	Moisture	Proteins	Fats	Ash	Fiber
SP8	9.10 ± 0.07^{A}	5.99 ± 0.07^{C}	0.89 ± 0.01^{B}	9.23 ± 0.05^{E}	34.77 ± 0.07^{AB}
SP17	8.27 ± 0.04^{B}	6.85 ± 0.06^{B}	0.82 ± 0.03^{C}	8.56 ± 0.05^{F}	34.92 ± 0.02^{A}
NH	7.16 ± 0.04^{D}	7.44 ± 0.03^{A}	0.40 ± 0.05^{E}	19.18 ± 0.03^{B}	21.88 ± 0.04^{E}
NS	6.37 ± 0.04^{E}	3.46 ± 0.04^{F}	1.21 ± 0.01^{A}	20.22 ± 0.03^{A}	34.60 ± 0.07^{B}
BH	4.37 ± 0.01^{F}	5.34 ± 0.01^{D}	0.59 ± 0.06^{D}	15.29 ± 0.09^{C}	26.93 ± 0.03^{D}
BS	7.83 ± 0.03^{C}	4.08 ± 0.03^{E}	0.35 ± 0.05^{F}	11.58 ± 0.06^{D}	32.46 ± 0.03^{C}

All values are expressed as mean ± SEM for sorghum and rice varieties. Values followed by same superscript letter along a column do not differ significantly ($p>0.05$ analyzed by ANOVA AND Tukey's post hoc test). Key: **SP8 -** Sorghum plot 8, **SP17** - Sorghum plot 17, **NH-**Nerica husk, **NS** - Nerica straw, **BH** - Basmati husk, **BS** - Basmati straw.

4.3 The quantity of structural carbohydrates generated from two different varieties of sorghum and rice treated with low concentrations of sulphuric acid (1.2%w/w and 2.25%w/w)

Table 4.2 shows statistical comparison of the quantity of structural carbohydrates from two different varieties of sorghum and rice at natural state (untreated biomass) and at low concentrations of Sulphuric acid. The quantity levels of the structural carbohydrates of biomass at natural state (untreated biomass), had cellulose levels in sorghum and rice varieties in this study ranging between 32% to 39% while the levels of hemicellulose across the biomass varieties ranged between 26% to 31%. The lignin content in rice husk used in this study were 15% for NH and 18% for BH, the rest of the biomasses in the study ranged between 7% to 11%.

The dilute acid pre-treatment exposed the cellulose for enzyme digestibility in this study, as shown in table 4.2 below, there was increased concentration levels of cellulose on dilute acid pre-treatment as compared to cellulose from untreated biomass. The treatment of biomass with sulphuric acid resulted in a note worth variation in SP8 and BS on treatment with 1.2%(w/w) and 2.25%(w/w). The other biomass varieties SP17, NS, NH, BH had no significant difference on the cellulose levels when treated with either of the 1.2%(w/w) or 2.25%(w/w) dilute acid concentration. BS treated with 2.25%(w/w) dilute sulphuric acid had the highest cellulose levels of 58.54% and NH and BH without dilute acid pre-treatment had the lowest cellulose level.

In this study the hemicellulose from sorghum and rice straws biomass varieties seemed to increase significantly regardless of the acid concentration. There was a significant difference among the varieties used in this study on the hemicellulose levels treated with different

percentages of acid concentration (1.2%(w/w) and 2.25%(w/w)) while the hemicellulose levels of NH had no significant difference on treatment with either 1.2%(w/w) or 2.25%(w/w) dilute sulphuric acid concentration.

Dilute sulfuric acid pre-treatment can interfere with lignin concentration and enhance cellulose susceptibility to enzymatic hydrolysis, but it does not completely dissolve or remove all the lignin. It can cause partial structure modification or solubilization of lignin components.The most significant compositional change among the biomass varieties was in lignin. After subjecting the biomass varieties to dilute acid pre-treatment, the lignin levels significantly reduced at both 1.2%(w/w) and 2.25%(w/w) concentration. At acid concentration 2.25%(w/w) all the biomass varieties, SP8, S17, NH, NS, BH, BS had extreme disruption on the lignin leading to lowest levels in comparison to 1.2%(w/w) concentration.

The removal of other constituents such as the lignin ash, resulted to a noticeable rise in the percentage of cellulose in comparison to the untreated feedstock. Thus, the enhanced conversion of cellulose would lead to an enhanced sugar accessibility in the hydrolysate.

Table 7.2:Quantity of structural carbohydrates across the selected agrowastes biomass in Percentages

Biomass	Cellulose	Hemicellulose	Lignin
SP8 untreated	35.24 ± 0.20^{G}	26.15 ± 0.05^{E}	7.68 ± 0.03^{E}
SP8 1.2	53.77 ± 0.33^{E}	26.44 ± 0.21^{E}	3.22 ± 0.02^{G}
SP8 2.25	56.18 ± 0.16^{BC}	29.43 ± 0.31^{BC}	2.83 ± 0.04^{HI}
SP17 Untreated	34.93 ± 0.87^{G}	26.72 ± 0.04^{E}	7.52 ± 0.13^{E}
SP17 1.2	54.54 ± 0.25^{DE}	26.71 ± 0.18^{E}	4.35 ± 0.13^{F}
SP17 2.25	55.03 ± 0.08^{D}	28.93 ± 0.3^{BC}	3.19 ± 0.04^{G}
NS Untreated	38.52 ± 0.88^{F}	26.76 ± 0.04^{E}	11.31 ± 0.11^{C}
NS 1.2	56.15 ± 0.34^{BC}	28.46 ± 0.20^{C}	3.03 ± 0.08^{GH}
NS 2.25	56.97 ± 0.07^{B}	29.55 ± 0.12^{B}	2.61 ± 0.03^{IJKL}
NH Untreated	32.14 ± 0.60^{H}	27.40 ± 0.10^{DE}	15.27 ± 0.11^{B}
NH 1.2	54.30 ± 0.33^{DE}	29.25 ± 0.13^{BC}	2.63 ± 0.04^{IJK}
NH 2.25	54.80 ± 0.25^{DE}	29.47 ± 0.08^{B}	2.37 ± 0.01^{L}
BH Untreated	32.32 ± 0.22^{H}	29.40 ± 0.08^{B}	18.81 ± 0.20^{A}
BH 1.2	54.42 ± 0.20^{DE}	28.96 ± 0.22^{BC}	2.64 ± 0.01^{IJK}
BH 2.25	55.12 ± 0.04^{CD}	31.68 ± 0.10^{A}	2.56 ± 0.01^{JKL}
BS Untreated	39.74 ± 0.36^{F}	26.03 ± 0.06^{E}	9.22 ± 0.12^{D}
BS 1.2	56.52 ± 0.26^{B}	28.21 ± 011^{CD}	2.71 ± 0.01^{IJ}
BS 2.25	58.54 ± 0.26^{A}	29.36 ± 0.14^{B}	2.43 ± 0.02^{KL}

Results are expressed as mean ±SEM for three triplicates. Analysis was carried out by ONE Way ANOVA followed by Tukey's post hoc. Groups sharing superscripts letter are not statistically different (P.>0.05). Key: **SP8 1.2-** Sorghum plot 8 1.2%(w/w), **SP8 2.25**-Sorghum plot 8 2.25%(w/w), S**P17 1.2**- Sorghum plot 17 1.2%(w/w), **SP17 2.25**- Sorghum plot 17 2.25%(w/w)**NH 1.2-**Nerica husk 1.2%(w/w), **NH 2.25**-Nerica husk 2.25, **NS 1.2**- Nerica straw 1.2%(w/w), **NH 2.25**-Nerica Straw 2.25%(w/w), **BH 1.2**-Basmati husk 1.2%(w/w), **BH 2.25**-Basmati husk 2.25%(w/w), **BS 1.2**-Basmati straw 1.2%(w/w), **BS 2.25**-Basmati straw 2.25%(w/w).

4.4 The levels of the by-products and hydroxylates of selected agro-waste biomass.

Figure 4.1, depicts the levels of HMF and furfural after detoxification. It is observed that 5-HMF and furfural levels are increasing with added acid concentration across almost all biomass substrates except for furfural levels in Nerica straws. Furfural levels in Nerica straws treated

with 1.2%(w/w) acid concentration was 0.4233% while furfural levels of Nerica straws treated with 2.25%(w/w) was 0.3966%. Sorghum plot 8 had 0.48% furfural in substrates treated with 1.2% conc of sulphuric acid and 0.49% in SP08 treated with 2.25%(w/w) acid concentration. This values of less than 1% were observed in all biomass varieties for both HMF and Furfural after pretreatment with 1.2%(w/w) and 2.25%(w/w) acid. The levels of HMF in all the biomass regardless of the acid concentration were lower in percentage to furfural levels.

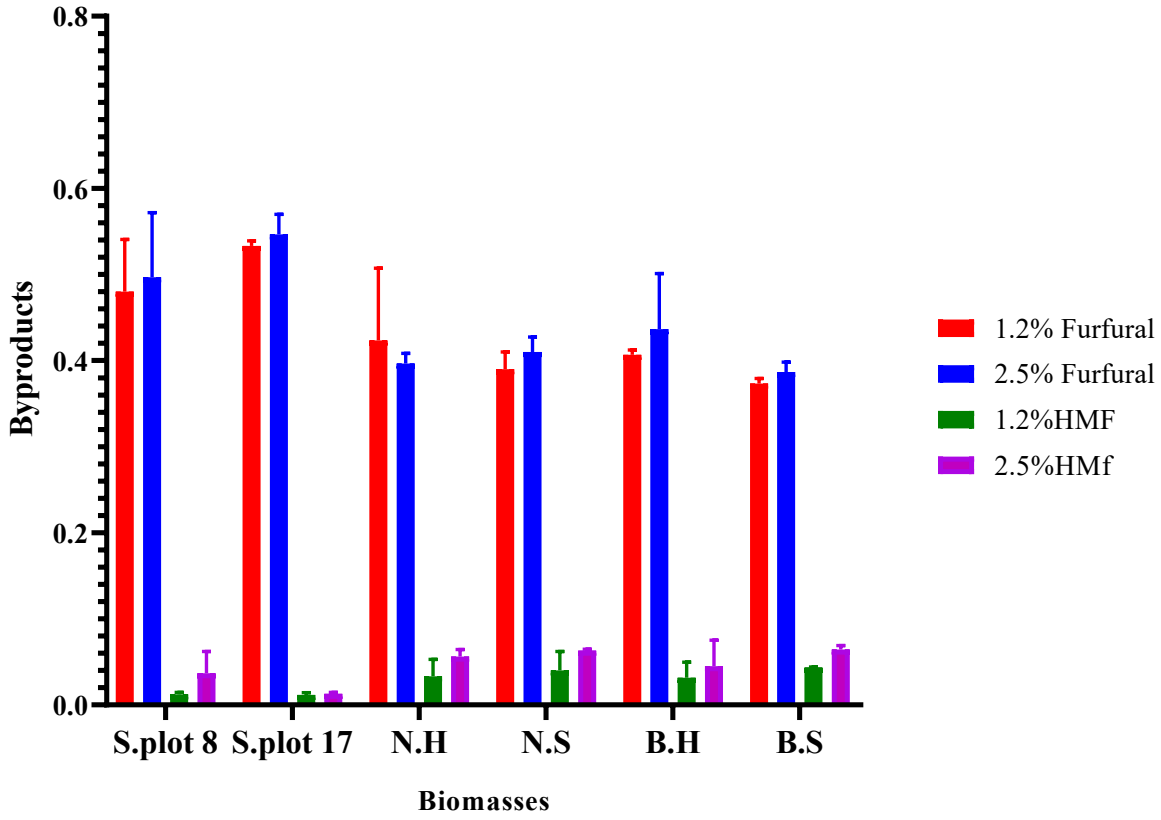

Figure 7.1: Degradative products after detoxification
Key: **S. Plot 8 -** Sorghum from plot 8, **S**. **Plot 17** – Sorghum from plot 17, **N.H -**Nerica husk, **N.S -** Nerica straw, **B.H** -Basmati husk, **B.S** -Basmati straw.

Pre-treatment with 1.2%(w/w) sulphuric acid resulted in formation of HMF which upon detoxification the presence was still detected. HMF values in SP8 and SP17 were statistically similar ($p>0.05$). Further, in the rice biomass varieties similar plant parts were comparable in yields statistically, NH and BH were comparable so is BS and NS ($P>0.05$) as shown in figure 4.2.

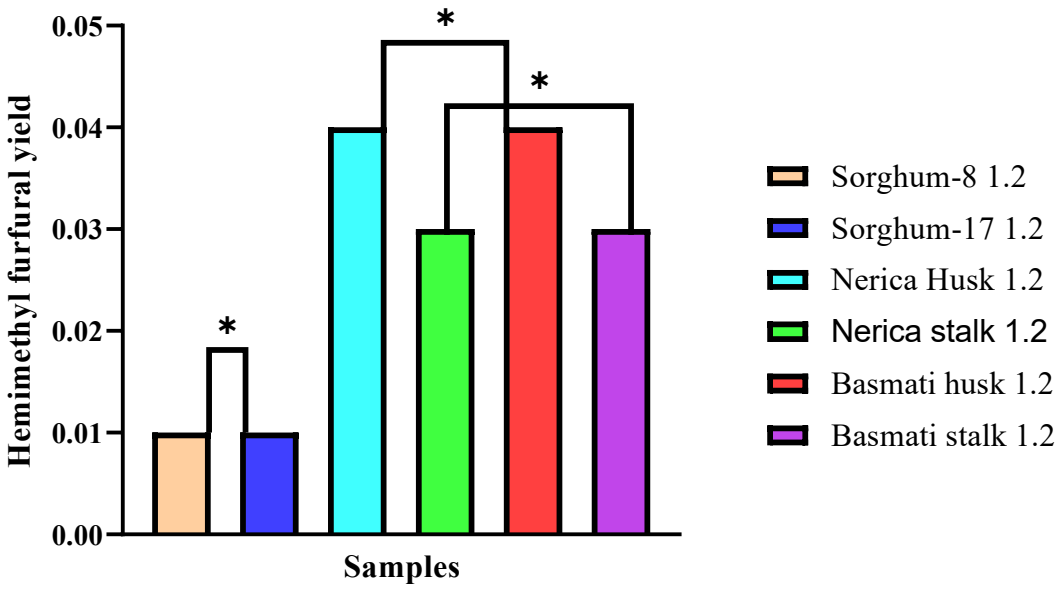

Figure 7.2: The percentage HMF at the concentration of 1.2%(w/w). Asterisk-designated bars are statistically comparable. The independent t-test was used for comparisons when p>0.05.

The yields of HMF in biomass pre-treated with 2.25%(w/w) sulphuric acid had a different trend in comparison to biomass treated with 1.2%(w/w) sulphuric acid. The rice biomass varieties regardless of plant part (Husk or Straw) were statistically comparable. The sorghum varieties in this our study were statistically incomparable to each other (P>0.05) as shown in figure 4.2. Further, SP8, SP17 were statistically incomparable to NH, NS, BH and BS.

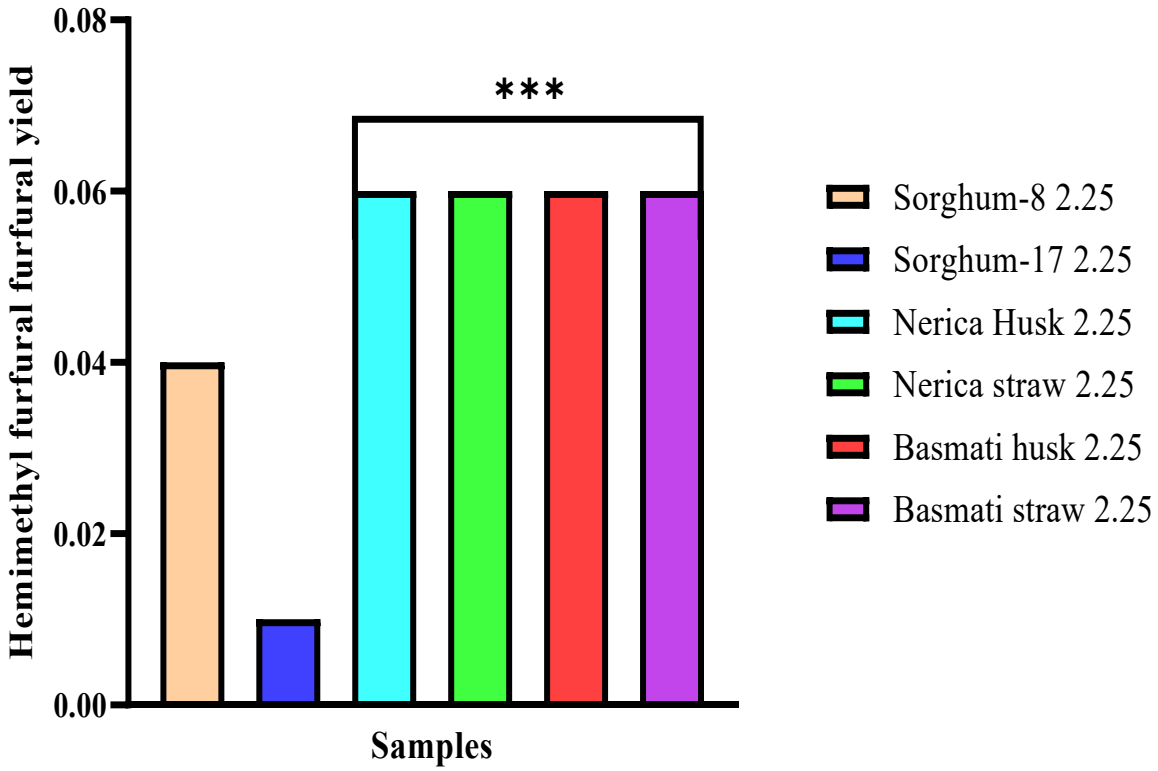

Figure 7.3: HMF % yield comparison after 2.25% pre-treatment and detoxification. Bars with asterisks are statistically similar. The independent t-test was used for comparisons when $p>0.05$.

Furfural yields after pre-treatment with1.2%(w/w) in sorghum varieties were statistically different. SP8 and SP17 were statistically incomparable ($p>0.05$). The rice biomass was comparable to similar rice plant part, NH was statistically similar to BH. Further NS was statistically comparable to BS ($P>0.05$). This was depicted in figure 4.3.

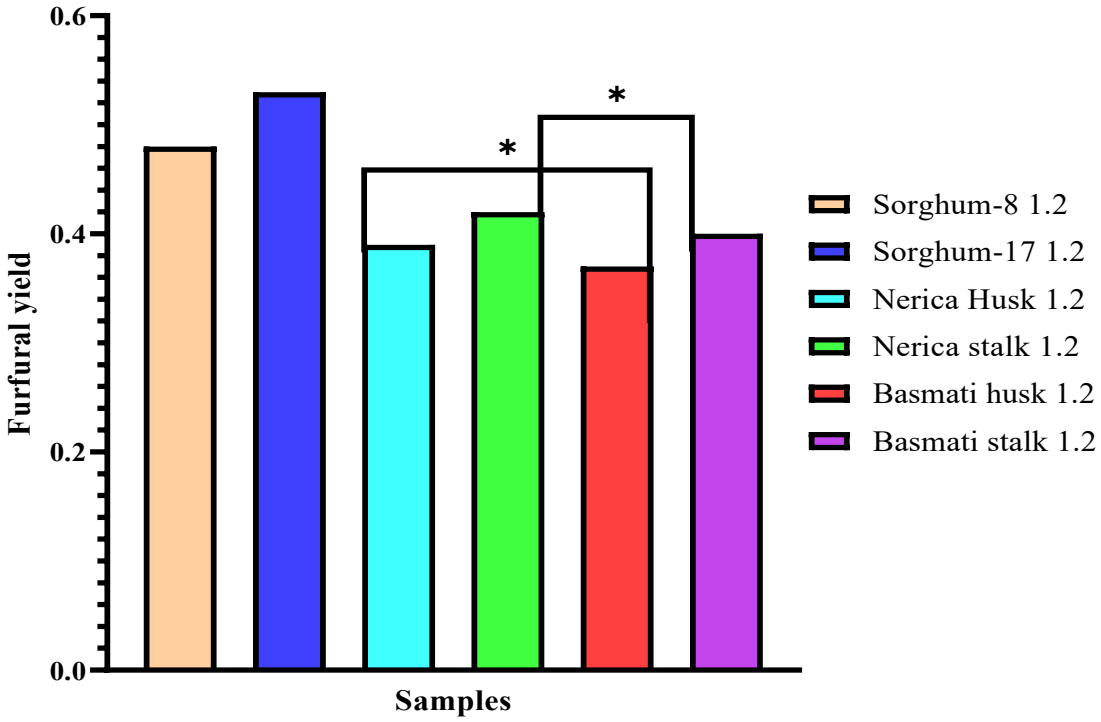

Figure 7.4: Percentage Furfurals yields comparison after 1,2%(w/w) pre-treatment and detoxification. Bars with asterisks are statistically similar. Comparisons were carried out using independent t-test with p>0.05.

In treatment with 2.25%(w/w) sulphuric acid, furfural levels in the rice biomass were statistically comparable regardless of plant part. NH, NS, BS, BH were statistically similar (P>0.05). Further the furfural yields in sorghum varieties was statistically different from each other, SP8 and SP17 were SP8 and SP17 were statistically incomparable (P>0.05).

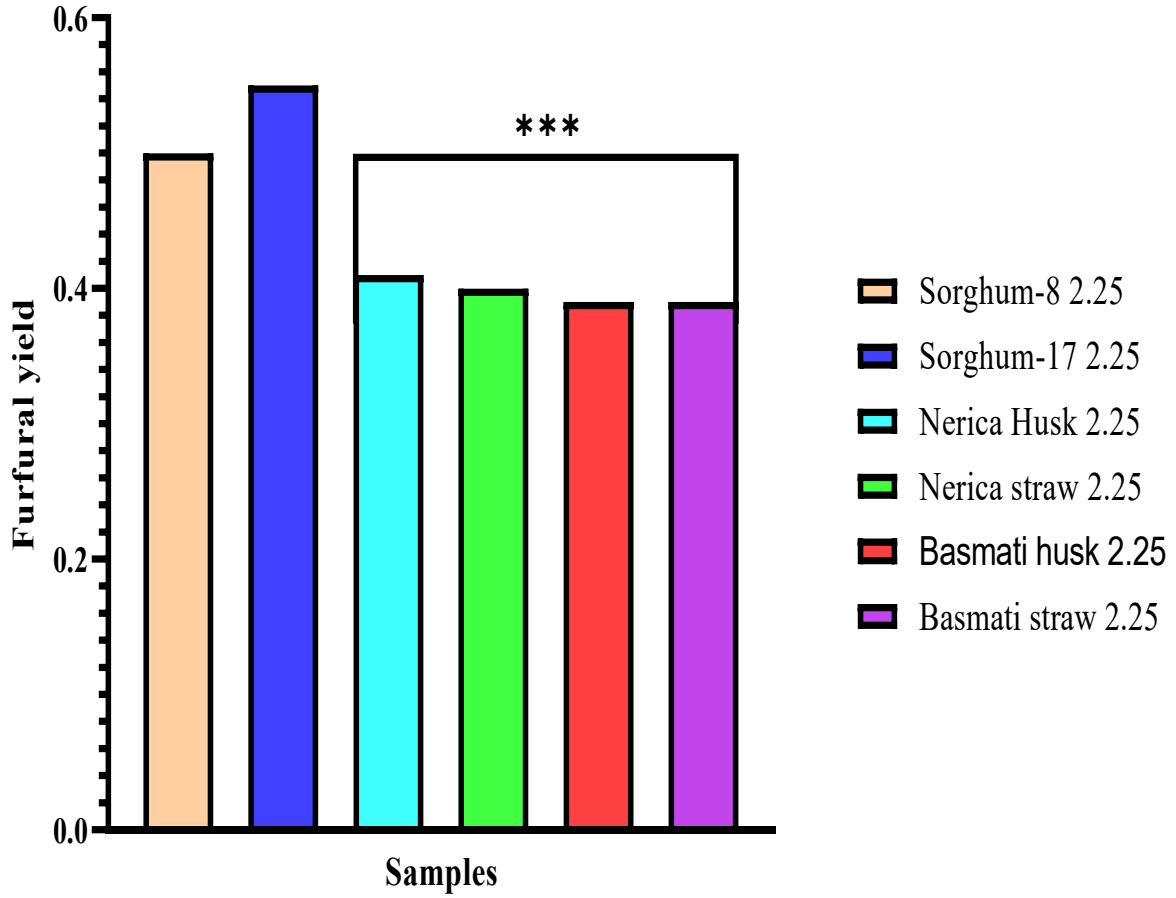

Figure 7.5: Percentage Furfurals yields comparison after pre-treatment and detoxification at the concentration of 2.25%(w/w). Bars with asterisks are statistically similar. Comparisons were carried out using independent t-test with $p>0.05$.

4.5 Simple sugars in the hydrolysates of the selected sorghum and rice varieties

Following diluted acid pre-treatment, enzyme saccharification occurs. Cellulose is transformed into glucose and other simple sugars in this step, and the amounts of these substances were measured using HPLC using pertinent standards (Table 4.3). There was no discernible variation observed in the glucose levels across all pre-treated biomass types treated with 1.2%(w/w) acid except for SP17 which had a glucose level of 12.8%(w/v) as compared to the other concentrations that ranged between 21%(w/v) and 26%(w/v). The biomass varieties pre-treated with 2.25%(w/w) acid conc had a similar trend, the varieties SP8 2.25, SP17 2.25, NH 2.25,

NS 2.25, BS 2.25, BH 2.25 had no significant difference on the glucose levels. The glucose levels ranged between 29% (w/v) and 32%(w/v). There was a significant difference on the Xylose and Sucrose sugar levels between all the biomass varieties pre-treated with 1.2%(w/w) acid concentration and 2.25%(w/w) acid concentration while on comparison to maltose sugar levels the opposite was observed, there was no significant difference among all the biomass varieties pre-treated with either 1.2%(w/w) or 2.25%(w/w) concentration except for BH and S8 varieties that had a significant difference on treatment with either of the acid concentration. The maltose level after hydrolysis had the BH biomass that had been pre-treated with 1.2%(w/w) acid concentration having least amounts of 1.13%(w/v) while BH pre-treated with 2.25%(w/w) acid concentration having the highest maltose level of 2.5%(w/v). There was no significant difference in mannose and arabinose levels between acid pre-treated biomass with 1.2%(w/w) or 2.25%(w/w) in sorghum plot 17 biomass. Biomass pre-treated with 2.25%(w/w) sulphuric acid with subsequent enzyme hydrolysis produced the highest xylose levels against biomass pre-treated with 1.2%(w/w) sulphuric acid followed by enzyme saccharification in the biomasses used in this study.

Table 7.3 Simple sugars in the hydrolysates of selected sorghum and rice varieties in Percentages(w/w)

Agro-waste	Glucose	Xylose	Mannose	Maltose	Arabinose	Sucrose
SP8 1.2	22.17±1.03^{B}	11.00±1.00^{B}	2.50±0.32^{C}	1.17±0.13^{C}	2.33±0.09^{B}	1.83±0.30^{CD}
SP8 2.25	29.29±0.9^{A}	25.33±1.45^{A}	5.23±0.79^{B}	2.47±0.23^{A}	4.67±0.62^{A}	3.27±0.26^{AB}
SP17 1.2	12.80±0.65^{C}	9.72±0.44^{B}	1.87±0.12^{C}	1.20±0.20^{BC}	2.30±0.21^{B}	1.17±0.15^{D}
SP17 2.25	13.86±0.67^{C}	24.53±1.45^{A}	2.83±0.19^{C}	2.33±0.35^{AB}	2.73±0.38^{B}	2.37±0.15^{BC}
N.H 1.2	26.73±2.2^{AB}	11.37±1.23^{B}	2.77±0.20^{C}	1.40±0.25^{AC}	2.43±0.03^{B}	1.93±0.29^{CD}
N.H 2.25	32.33±0.6^{A}	22.13±0.59^{A}	6.50±0.31^{AB}	2.47±0.20^{A}	5.57±0.18^{A}	3.93±0.18^{A}
N.S 1.2	21.30±1.02^{B}	11.33±1.20^{B}	2.87±0.12^{C}	1.37±0.12^{AC}	2.47±0.03^{B}	1.97±0.26^{CD}
N.S 2.25	30.57±0.4^{A}	25.00±1.15^{A}	6.70±0.12^{AB}	2.20±0.21^{AC}	5.60±0.15^{A}	4.13±0.23^{A}
B.H 1.2	22.62±1.08^{B}	11.33±1.45^{B}	2.53±0.13^{C}	1.13±0.12^{C}	1.97±0.07^{B}	2.13±0.15^{BD}
B.H 2.25	31.60±1.2^{A}	22.00±1.00^{A}	6.63±0.12^{AB}	2.50±0.35^{A}	5.87±0.27^{A}	4.00±0.10^{A}
B.S 1.2	22.93±0.77^{B}	10.00±1.00^{B}	2.30±0.10^{C}	1.23±0.20^{BC}	2.30±0.31^{B}	2.20±0.10^{BD}
B.S 2.25	30.30±0.4^{A}	25.60±1.50^{A}	6.80±0.21^{A}	2.33±0.15^{AB}	5.43±0.12^{A}	4.23±0.35^{A}

Results are expressed as mean ±SEM for three triplicates. Analysis was carried out by ONE Way ANOVA followed by Tukey's post hoc. Groups sharing superscripts letter are not statistically different (P.>0.05). Key: **SP8 1.2-** Sorghum plot 8 1.2%(w/w), **SP8 2.25**-Sorghum plot 8 2.25%(w/w), S**P17 1.2**- Sorghum plot 17 1.2%(w/w), **SP17 2.25**- Sorghum plot 17 2.25%(w/w) **N.H 1.2-**Nerica husk 1.2%(w/w), **N.H 2.25**-Nerica husk 2.25(w/w), **N.S 1.2**- Nerica straw 1.2%(w/w), **N.H 2.25**-Nerica Straw 2.25%(w/w), **B.H 1.2**-Basmati husk 1.2%w/w), **B.H 2.25**-Basmati husk 2.25%(w/w), **B.S 1.2**-Basmati straw 1.2%(w/w), **B.S 2.25**-Basmati straw 2.25%(w/w).

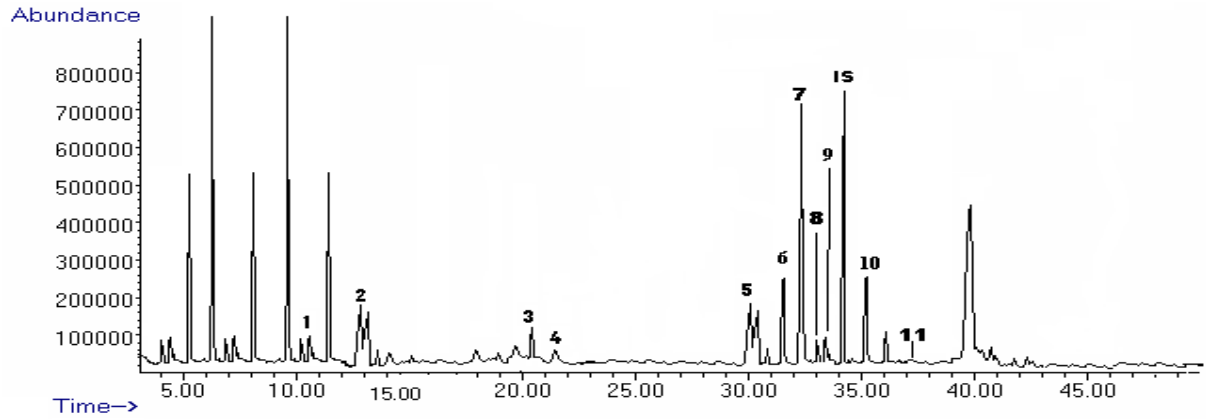

Figure 7.6: HPLC peaks of BH a after pre-treatment with 1.2% acid and enzyme saccharification. HPLC chromatograms from hydrolysates of BH sample a. The chromatograms show the separation of different simple sugars from BH. Peaks analogous to standards for fructose (1), arabinose (2), maltose (3), glucuronic acid (4), Mannose (5), xylose (6), glucose (7), cellobiose (8), D-galactose (9), sucrose (10), internal standard (IS), xylobiose (11).

Table 7.4: Detection and retention of different simple sugars in BH a 1.2% Hydrolysate

Peak No	Sugars	Retention Time (RT)	% Quality
1	Fructose	11.542	86.0
2	Arabinose	13.222	92.0
3	Maltose	22.059	90.0
4	Glucoronic acid	22.214	86.0
5	Mannose	30.008	90.0
6	Xylose	31.530	93.0
7	Glucose	32.245	98.0
8	Cellobiose	33.624	92.0
9	D-galactose	33.978	90.0
10	Sucrose	34.007	89.0
IS	Internal Standard	35.115	98.0
11	Xylobiose	37.253	67.0

Detection and retention (RT) of different simple sugars using reverse phase column and refractive index detector.

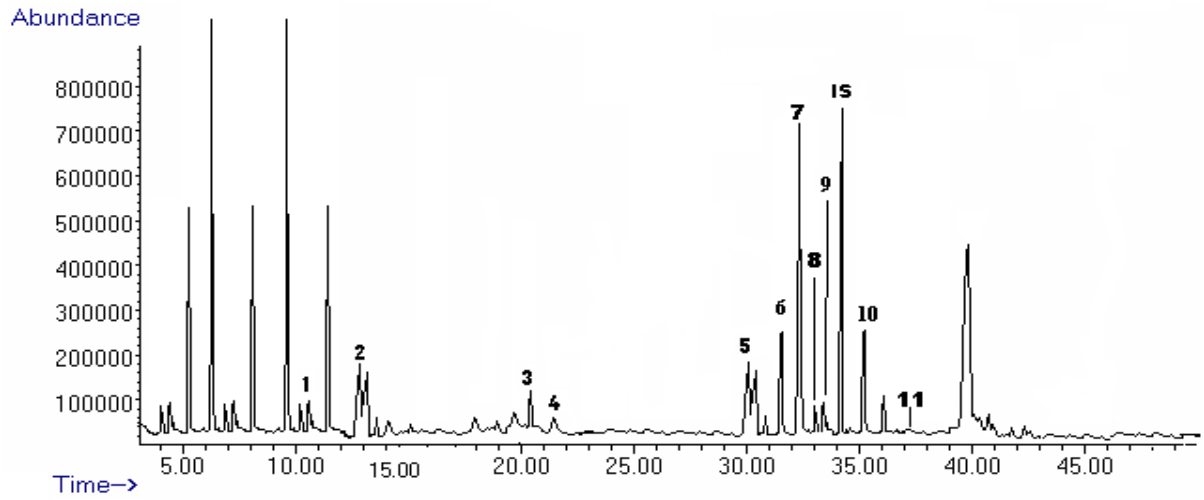

Figure 7.7: HPLC peaks of SP17 a after pre-treatment with 2.25% sulphuric acid. HPLC chromatograms showing the peaks of sugars of SP17a biomass after enzyme hydrolysis with cellulase enzyme from aspergillus niger. Peaks analogous to standards for fructose (1), arabinose (2), maltose (3), glucuronic acid (4), Mannose (5), xylose (6), glucose (7), cellobiose (8), D-galactose (9), sucrose (10), internal standard (IS), xylobiose (11).

Table 7.5: Detection and retention of different sugars of SP17 2.25%a Hydrolysate

Peak No	Sugars	Retention Time (RT)	% Quality
1	Fructose	11.884	85.0
2	Arabinose	13.232	92.0
3	Maltose	22.060	90.0
4	Glucoronic acid	22.114	87.0
5	Mannose	30.008	90.0
6	Xylose	31.526	93.0
7	Glucose	32.247	97.0
8	Cellobiose	33.629	93.0
9	D-galactose	33.897	90.0
10	Sucrose	34.014	91.0
IS	Internal Standard	35.121	98.0
11	Xylobiose	37.198	66.0

Detection and retention (RT) of different simple sugars using reverse phase column and refractive index detector

Glucose was the highest produced sugar from all the biomass varieties after 2.25%(w/w) dilute sulphuric acid pre-treatment and cellulase enzyme from *Aspergilus niger* hydrolysis. As depicted in figure 4.8, The Rice varieties had the highest glucose sugar levels with no significant difference and the sorghum varieties had the least glucose levels, there was a significant difference between SP8 and SP17 varieties. sorghum plot 8 had 25.4%(w/w) while sorghum plot 17 had 13.8%(w/w). The maltose and sucrose sugar levels after 2.25%(w/w) dilute acid pre-treatment showed no significant difference in levels among all the biomass varieties, they all ranged between 0.9%(w/w) to 3.5%(w/w). This trend was also observed after 1.2%(w/w) dilute sulphuric acid pre-treatment and cellulase enzyme hydrolysis as depicted in table 4.3.

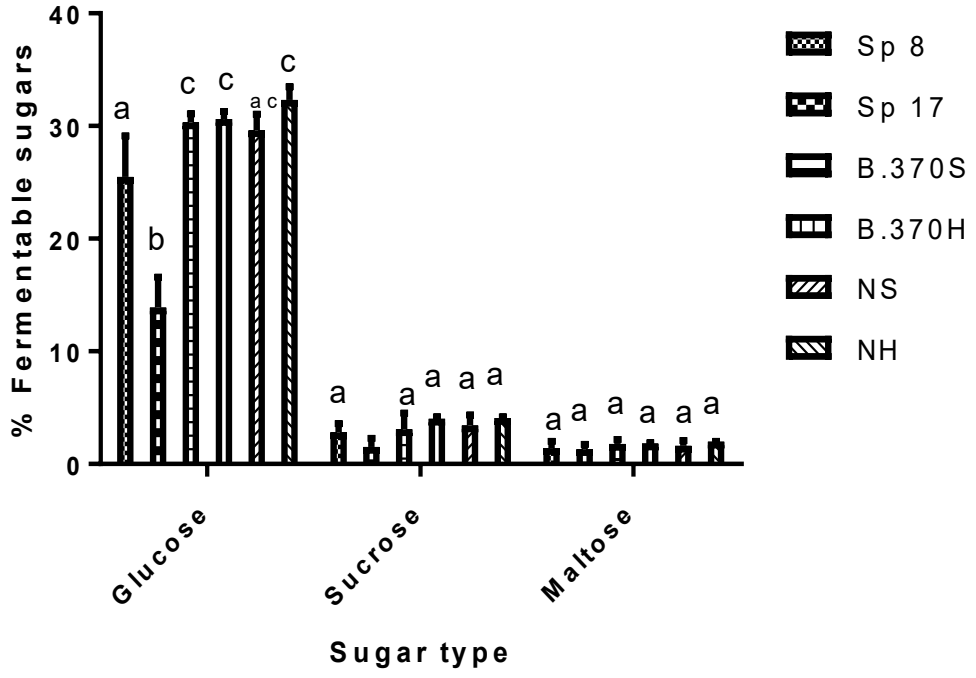

Figure 7.8: Fermentable sugars yielded in percentages(v/v) when pre-treated biomass with 2.25%(w/w) sulphuric acid was subjected to enzymatic hydrolysis. Values are presented as Mean ± SD, n=3Z means with similar sugar type concentration followed by the same letter(s) were not significantly different (P≤0.0001). Values are based on 2.25%(w/w) acid pre-treatment.

4.6 The total ethanol yield of the selected agro-waste biomass.

The ethanol yields produced after fermentation with *Saccharomyces cerevisae* at 30°C, 150rpm for 168 hours is presented in Table 4.6 below. The outcomes show the yields of bioethanol manufactured from different biomasses that were pretreated with 1.2%(w/w) sulphuric acid had lower yields of ethanol as compared to yields from 2.25%(w/w) acid pretreated substrates.

There was a substantial difference on ethanol produce of the various biomasses on pre-treatment with acid concentrations as shown in table 4.6 below. The results indicate that the Rice husk varieties produced the highest amount of ethanol content from 10% biomass loading of about 8.89%(w/v) ethanol content after 2.25%(w/w) dilute acid pre-treatment. The biomass type has a significant effect on ethanol yield, Sorghum plot 17 produced the least amount of ethanol among the biomasses used with 5.80%(v/v) ethanol content while Basmati husk had the highest yield of 8.89%(v/v) after 2.25% acid pre-treatment.

Table 7.6: Total ethanol yield of selected agro-waste at different acid concentrations in percentages(v/v)

Biomass	Ethanol yield	Biomass	Ethanol yield	Biomass	Ethanol yield
SP8 1.2	5.48 ± 0.31^{DE}	NH 1.2	4.79 ± 0.09^{E}	BH 1.2	6.12 ± 0.14^{CD}
SP8 2.25	7.59 ± 0.22^{AB}	NH 2.25	8.43 ± 0.12^{A}	BH 2.25	8.89 ± 0.24^{A}
SP17 1.2	5.39 ± 0.19^{DE}	NS 1.2	5.27 ± 0.06^{DE}	BS 1.2	6.12 ± 0.32^{CD}
SP17 2.25	5.80 ± 0.23^{CDE}	NS 2.25	8.05 ± 0.29^{A}	BS 2.25	8.41 ± 0.29^{A}

Values are expressed as Mean± SEM for triplicates. One Way ANOVA was carried out in followed by Tukey's post hoc. Values with different superscripts are statistically different.

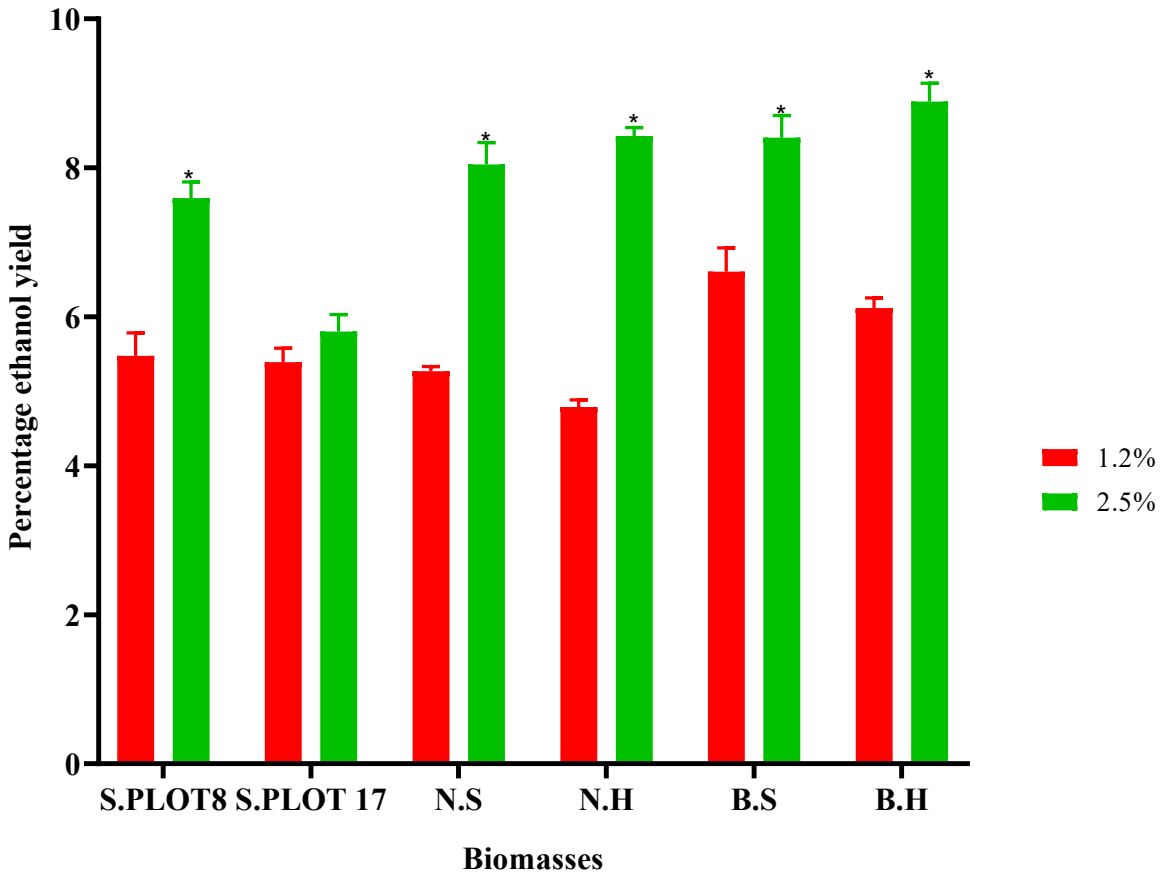

Figure 7.9: Ethanol percentage yields(v/v) from Agro-wastes biomass. **Key: S**. **P**lot **8 - Sorghum from plot 8,** S. **P**lot **17 –** Sorghum from plot 17**, N.H -**Nerica husk**, N.S** - Nerica straw**, B.H -**Basmati husk**,** B.S -Basmati straw**.**

The ethanol yield that was obtained from the different biomass types varied significantly following pre-treatment with varying concentrations of dilute sulfuric acid. Both the type of biomass and the acid concentration used during pre-treatment were found to have a significant influence on ethanol production as shown in figure 4.9 above. Among the tested concentrations, treatment with 2.25%(w/w) sulphuric acid produced the highest ethanol levels across all biomass types, suggesting its superior effectiveness in enhancing fermentable sugar availability. Furthermore, a comparison t-test conducted at the 2.25%(w/w) acid level to identify the most suitable biomass for ethanol production among those evaluated in the study. The results confirmed that biomass type has a considerable effect on the ethanol yield,

underscoring the importance of selecting both the appropriate feedstock and pre-treatment conditions to maximize bioethanol production efficiency.

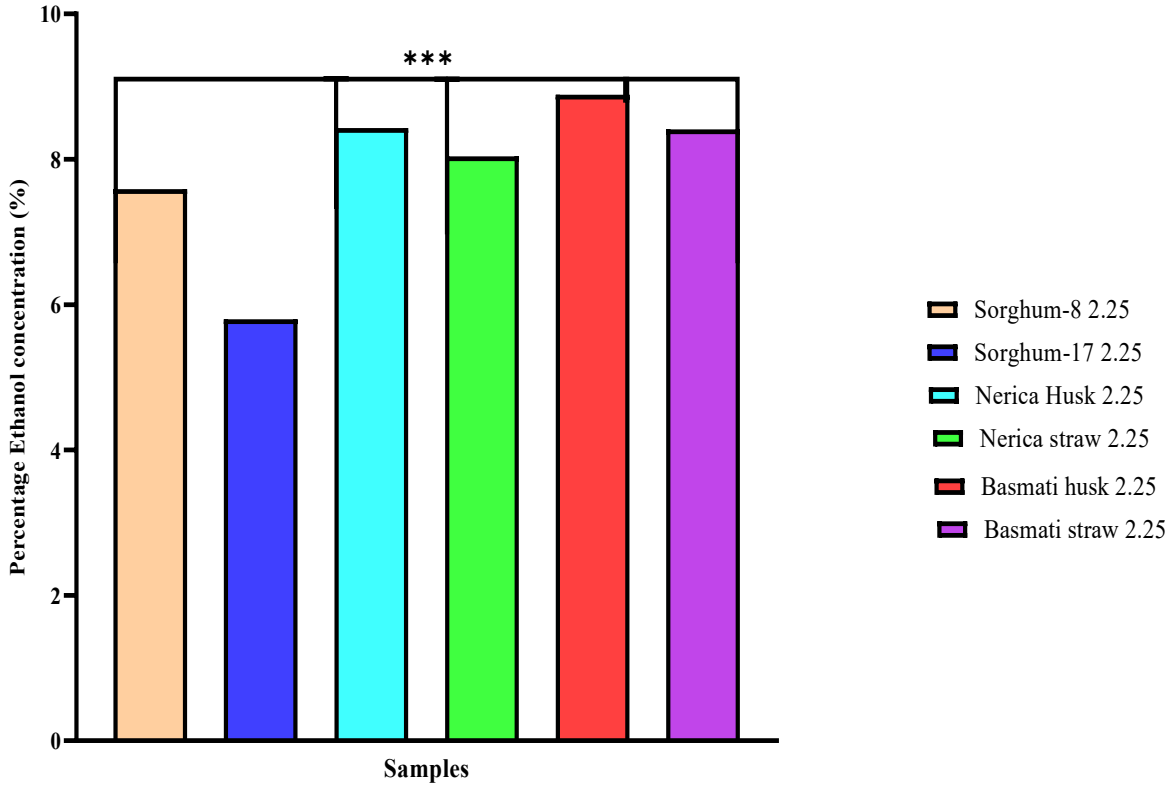

Figure 7.10: Comparison of percentage/v ethanol yields across biomass treated with 2.25% acid concentration. Bars with asterisks are statistically similar. Comparisons were carried out using independent t-test with p>0.05.

Ethanol yields from biomass samples pre-treated with 2.25%(w/w) sulfuric acid varied significantly across the different biomass types. As illustrated in figure 4.10 above, the yield from SP08 was statistically comparable ($p > 0.05$) to those from NH, NS and BS, as determined by independent *t*-tests. However, the ethanol yield from SP8 was significantly different ($p < 0.05$) from that of SP17, which exhibited the lowest ethanol output among the tested varieties at 2.25%(w/w) acid concentration. These results highlight the influence of biomass variety on ethanol production efficiency following uniform pre-treatment conditions.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

There are various steps that should be considered for improving bioethanol production, key among them include selection of suitable biomass, selection of suitable micro-organisms and process optimization. To achieve high yields of ethanol, favorable pre-treatment and hydrolysis processes are to be developed (Broda *et al*., 2022). Selection of those processes require appropriate choice of acid, right concentration of acid and favorable environmental conditions for proper growth and secretion of enzymes (Studer *et al*., 2011).

The Proximate content offers a platform for comparison between various species, plant sections, and cultivation circumstances and gives a solid first impression of the relative nutritional content and usability of an agricultural commodity. The proximate composition of rice husk, rice straws and sorghum stalk analyzed in this study had values that are close and in range with values obtained from other energy crops such as corn and sugarcane bagasse. This data is in agreement with results from Korotkova *et al*., (2016) and Wannapeera *et al*., (2008). Straws from the Basmati Rice varieties in this study had moisture content values that were comparable to the results obtained from untreated rice straws biomass in the research on proximate composition profiling by Santiago *et al* (2016). Santiago *et al* (2016) found moisture content of 8% in their untreated rice straws while the moisture content in BS for this study was found to be 7.83% and 6.37% from NS. The low moisture content observed in this study, ranging from the lowest of 4.38% in BH to 9.10% in SP8 from the analyzed feedstock in this study indicate that the various biomass can burn off easily as a source of heat, besides that, moisture content below 50% also affect downstream process by swelling the biomass hence increasing enzyme infiltration to the cellulose. The ash content of 19.18% in NH rice variety

and NS values of 20.22% in this study were similar to data from Binod *et al* (2010) in their review on rice straw and rice husk as a source of bioethanol production which got ash content values of 18.67% for the rice straws and 20.26% for the Rice husk. The low ash content obtained in sorghum and basmati straws and husk of this study indicate this varieties will yield lower residues as compared to Nerica rice straws and husk variety on complete combustion. In comparison to literature, the sorghum, rice straws and rice husks biomass varieties used in this study based on their nutritive value, present different potentials that may be exploited by bioenergy sector.

Structural composition of different biomass is an important analytical procedure since they determine the type of simple sugars to be expected after different pre-treatments and hydrolysis of biomass. While it is evident that compositional differences can be striking between different biomass types, there is also a considerable compositional unpredictability between different anatomical fractions of the same type of biomass. Structural carbohydrate values for rice straw biomass varieties for this study were in range with the Netherland Programme sustainable Biomass report (Bakker *et al*., (2013). Their cellulose values were between 28% to 41%, the hemicellulose values were between 21%-26% while Lignin content was between 9% to 23% on dry weight basis. The same trend was also observed, Cellulose content of between 28%-38% and Lignin contents ranging from 9-20% from Kuban state technological university on physical properties and chemical composition of rice husk and dust. This structural carbohydrate values were similar with data of the varieties of rice husk used in this study. The Lignin values, Cellulose and hemicellulose content in sorghum stalk varieties in this study showed similarities in value with data obtained by (Pimentel *et al*., 2017). Their hemicellulose values of the sorghum bagasse cultivars ranged between 28% -31% while their lignin values ranged between 5%-10% and their cellulose values ranged between 22%-31%. The slight

differences in values in this study could be affiliated to the plant genetics, growing conditions and the environmental factors of the region. Determination of biochemical components is very important in determining the suitability for various conversion processes as well as their economic feasibility. Among the three components, cellulose is the most affable to the production of biofuels like bioethanol by enzymatic hydrolysis followed by fermentation. Bioethanol can also be prepared from hemicellulose. The biochemical component was distinctively different from each other. The highest cellulose content from raw biomass varieties would make Basmati straws and Nerica rice straws biomass desirable for ethanol than sorghum varieties.

Dilute acid pre-treatment was a vital phase in conversion of the lignocellulosic substrate to ethanol in this study. The plant cell walls are very difficult to hydrolyze and the process of pre-treatment using dilute acid was important to lay bare hemicellulose and cellulose for subsequent enzymatic hydrolysis (Rocha-Meneses *et al*., 2017). Pretreatment attempts to reduce cellulose's crystallinity, breaking the lignin seal, increase biomass surface area, and eliminate the three primary structural carbohydrate units of lignocellulose, which are arranged into a complex structure that resists breakdown. Hemicellulose is easily hydrolysed to pentose’s and hexoses by acid treatment (Taherzadeh and Karimi 2007). In the current study, the use of dilute sulphuric acid improved the accessibility of cellulose polysaccharide which is useful in enzyme hydrolysis for glucose yields. Determination of the carbohydrate profile components were very important in determining the suitability for various conversion process as well as their economic feasibility. Among the three components cellulose was the most amenable to the production of bioethanol by enzymatic hydrolysis. The biochemical components among the biomass varieties used in this study were distinctively different. The highest cellulose content from BS biomass after pre-treatment with 2.25%(w/w) acid

concentration would make Basmati straw biomass the desirable feedstock for production of ethanol than Nerica Husk biomass pre-treated with 2.25%(w/w) acid concentration.

Enzyme hydrolysis is a key step in monosaccharide sugar production. Except for sorghum S17 with glucose level of 13.86% (w/w) all the other biomass varieties had glucose sugar levels of between 29%(w/w) to 32%(w/w) on biomass pre-treated with 2.25%(w/w) sulphuric acid. The detection of neutral sugars (pentoses) could have been components of hemicellulose. Timilsena, (2012) states that dilute acid pre-treatment affects the crystalline structure of lignin and the crystallinity of the hemicellulose hydrolysing it to pentoses and hexoses. It is also possible that during detoxification some of the monosaccharides produced were degraded in sorghum plot 17 hence the low levels of monosaccharides observed in the hydrolysate in this study. Kaur *et al*., 2023 states that over liming reduces the toxicity of the by-products formed during acid pre-treatment with the side effect of monosaccharide loss. This can further be explained by the results obtained in this study, before detoxification the cellulose content of the two sorghum varieties were not significantly different from each other. SP8 had a cellulose content of 56.4% while SP17 had cellulose content of 54.85% after 2.25%(w/w) acid pre-treatment. Despite comparable cellulose content among the sorghum varieties, significant differences in glucose yield were observed. This suggests that factors such as the high inhibitory products, lignin concentration and structure, cellulose crystallinity, hemicellulose composition, and pre-treatment effectiveness played a critical role in determining enzyme accessibility and hydrolysis efficiency (Deshavath *et al*., 2017) . These structural and chemical differences likely influenced how readily the cellulose was converted to glucose during enzymatic hydrolysis. The glucose sugar levels were much lower in SP17 with glucose levels of 13.86%(w/w) while SP8 had 29.29%(w/w) glucose level on samples pre-treated with 2.25%(w/w) acid concentration. This trend was also observed in 1.2%(w/w) acid pre-treatment on similar sorghum biomass.

Throughout the fermentation process, *Saccharomyces cerevisiae* utilized the glycolytic pathway to metabolize the glucose present in the substrates, producing pyruvate and subsequently converting it to ethanol under anaerobic conditions. The variation in ethanol yields in Basmati rice husk is an indication of a superior glucose content and less inhibitory materials. From this study, it was observed that the bioethanol yields from the various hydrolysates at pH 6, at 30˚C temperature for 120hrs of incubation the various biomasses gave different ethanol yields though not significantly different. This variation in respective of feedstock could be as a result of the inhibitory materials they contain. The low ethanol yield among the biomass feedstocks despite high yields of sugar could also be as a result of the type of yeast, this can significantly affect the yield since conventional yeast can only ferment glucose.

5.2 Conclusion

I. The proximate Composition and Structural carbohydrates analysis revealed considerable variability among the selected agro-wastes.

II. Among the two acid concentrations tested, 2.25%(w/w) sulphuric acid was found to be the most effective in improving the release of fermentable sugars, particularly glucose. 2.25%(w/w) concentration consistently resulted in enhanced cellulose levels across all biomass types, while also maintaining manageable levels of inhibitor formation.

III. There was no significant difference in ethanol yields in the rice varieties treated with similar dilute acid concentrations on the hand there was a significant difference between ethanol yields between the 2 sorghum types.

5.3 Recommendation

I. There is need to broaden the dilute acid pretreatment ranges beyond 1.2%w/w and 2.25%w/w for effective feedstock hydrolysis.

II. Ethanol yields of more than 8%(v/v) inhibit *Saccharomyces cerevisae* activity hence it is important to find a microbe that withstand higher ethanol yields

5.4 Recommendations for future studies

I. Ethanol yield should have been monitored and removed to hinder reaching the levels of ethanol concentration that inhibit *Saccharomyces cerevecea* activity.

II. Over liming as a detoxification process is applied to alleviate the harmfulness and reduce fermentation inhibitors though it has a consequence of degrading monosaccharide sugars which results in sugar loss that could otherwise increase the sugar concentration and ultimately increase the ethanol yields.

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APPENDICES

APPENDIX: HPLC Chromatograms and Peak Data

Figure 1: HPLC chromatogram of sample BH b 1.2% (w/w) showing separation of sugars.

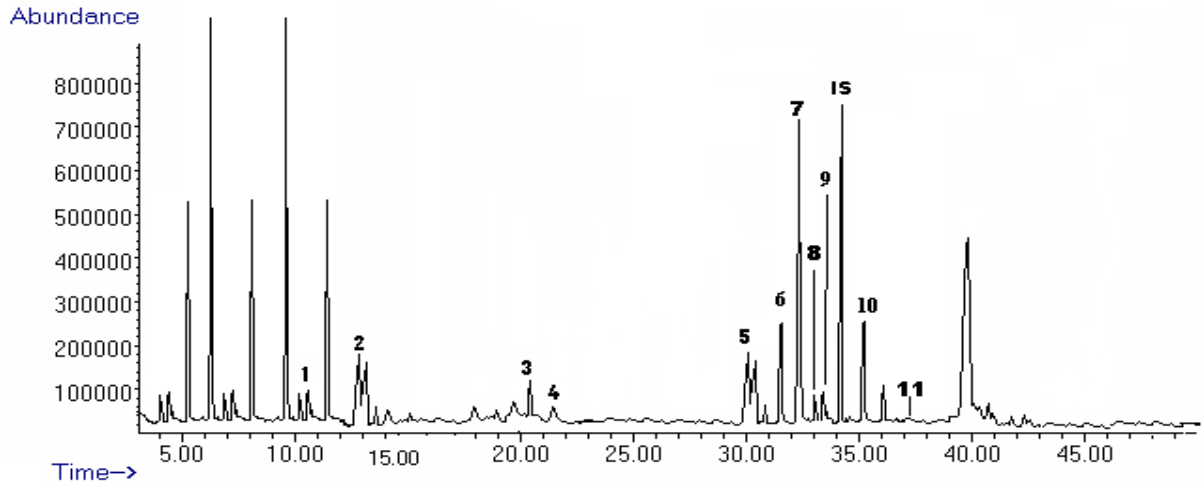

Table 1: HPLC results showing peak area and retention time for compounds detected in sample BH B 1.2% (w/w)

Peak No	Sugars	Retention Time(RT)	% Quality
1	Fructose	11.541	86.0
2	Arabinose	13.224	92.0
3	Maltose	22.059	90.0
4	Glucoronic acid	22.214	86.0
5	Mannose	30.008	90.0
6	Xylose	31.531	93.0
7	Glucose	32.245	98.0
8	Cellobiose	33.628	92.0
9	D-galactose	33.978	90.0
10	Sucrose	34.007	89.0
IS	Internal Standard	35.115	98.0
11	Xylobiose	37.254	67.0

Figure 2: HPLC chromatogram of sample BH a 1.2% (w/w) showing separation of sugars.

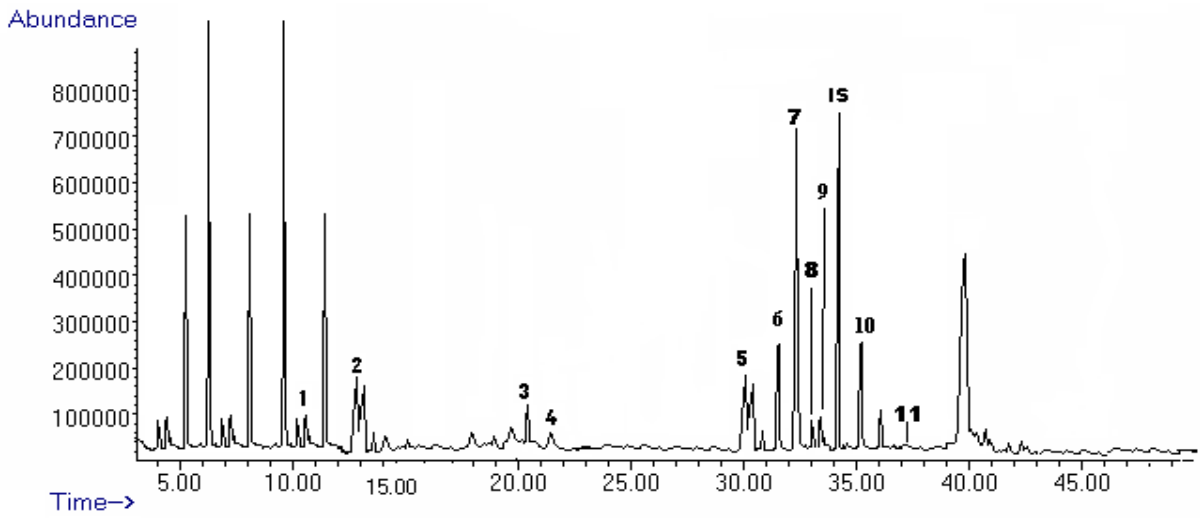

Table 2: HPLC results showing peak area and retention time for compounds detected in sample BH a 1.2% (w/w)

Peak No	**Sugars**	**Retention Time(RT)**	**% Quality**
1	Fructose	11.542	86.0
2	Arabinose	13.222	92.0
3	Maltose	22.059	90.0
4	Glucoronic acid	22.214	86.0
5	Mannose	30.008	90.0
6	Xylose	31.530	93.0
7	Glucose	32.245	98.0
8	Cellobiose	33.624	92.0
9	D-galactose	33.978	90.0
10	Sucrose	34.007	89.0
IS	Internal Standard	35.115	98.0
11	Xylobiose	37.253	67.0

Figure 3: HPLC chromatogram of sample SP 7 a 2.25% (w/w) showing separation of sugars.

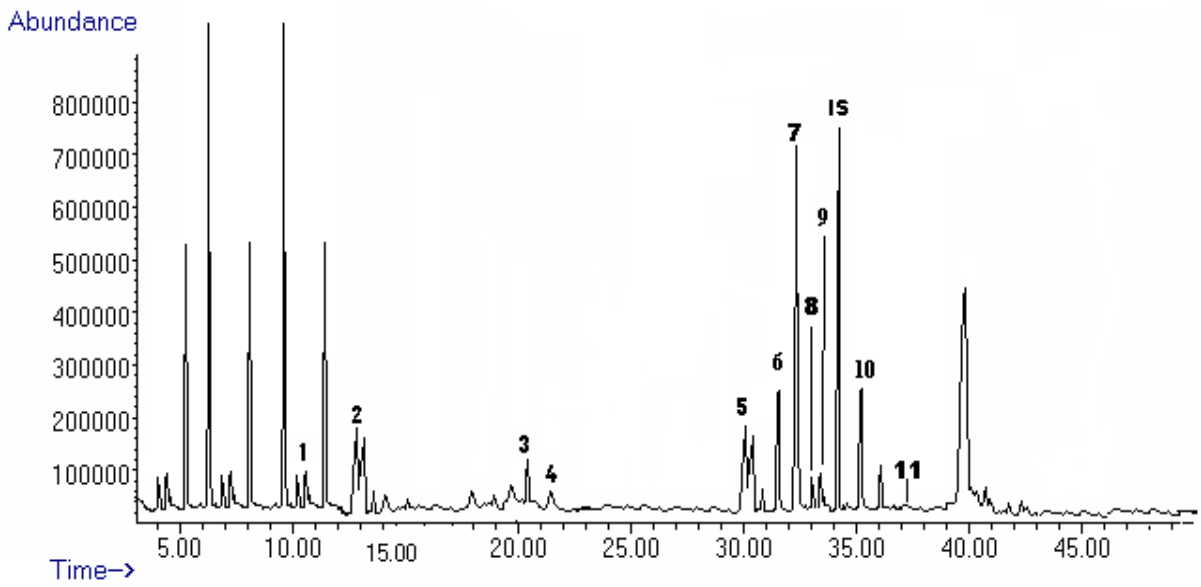

Table 3: HPLC results showing peak area and retention time for compounds detected in sample SP 7 a 2.25% (w/w)

Peak No	**Sugars**	**Retention Time(RT)**	**% Quality**
1	Fructose	11.884	85.0
2	Arabinose	13.232	92.0
3	Maltose	22.060	90.0
4	Glucoronic acid	22.114	87.0
5	Mannose	30.008	90.0
6	Xylose	31.526	93.0
7	Glucose	32.247	97.0
8	Cellobiose	33.629	93.0
9	D-galactose	33.897	90.0
10	Sucrose	34.014	91.0
IS	Internal Standard	35.121	98.0
11	Xylobiose	37.198	66.0

Figure 4: HPLC chromatogram of sample SP b 2.25% (w/w) showing separation of sugars.

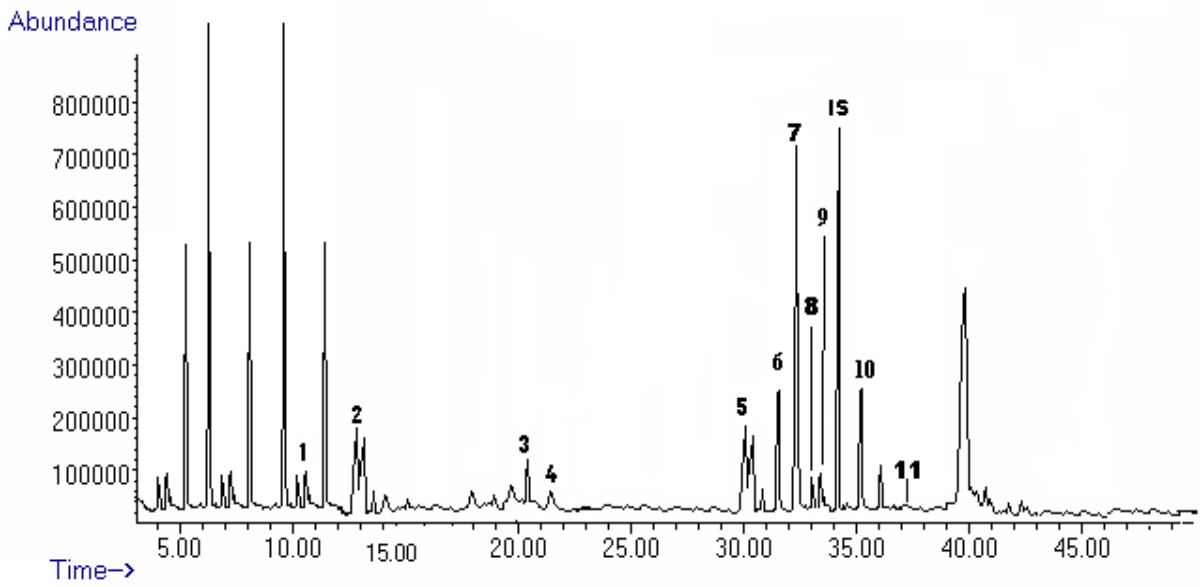

Table 4: HPLC results showing peak area and retention time for compounds detected in sample SP 7 b 2.25% (w/w)

Peak No	**Sugars**	**Retention Time (RT)**	**% Quality**
1	Fructose	11.541	86.0
2	Arabinose	13.224	92.0
3	Maltose	22.059	90.0
4	Glucoronic acid	22.214	86.0
5	Mannose	30.008	90.0
6	Xylose	31.531	93.0
7	Glucose	32.245	98.0
8	Cellobiose	33.628	92.0
9	D-galactose	33.978	90.0
10	Sucrose	34.007	89.0
IS	Internal Standard	35.115	98.0
11	Xylobiose	37.254	67.0

Figure 5: HPLC chromatogram of sample NS b 2.25% (w/w) showing separation of sugars.

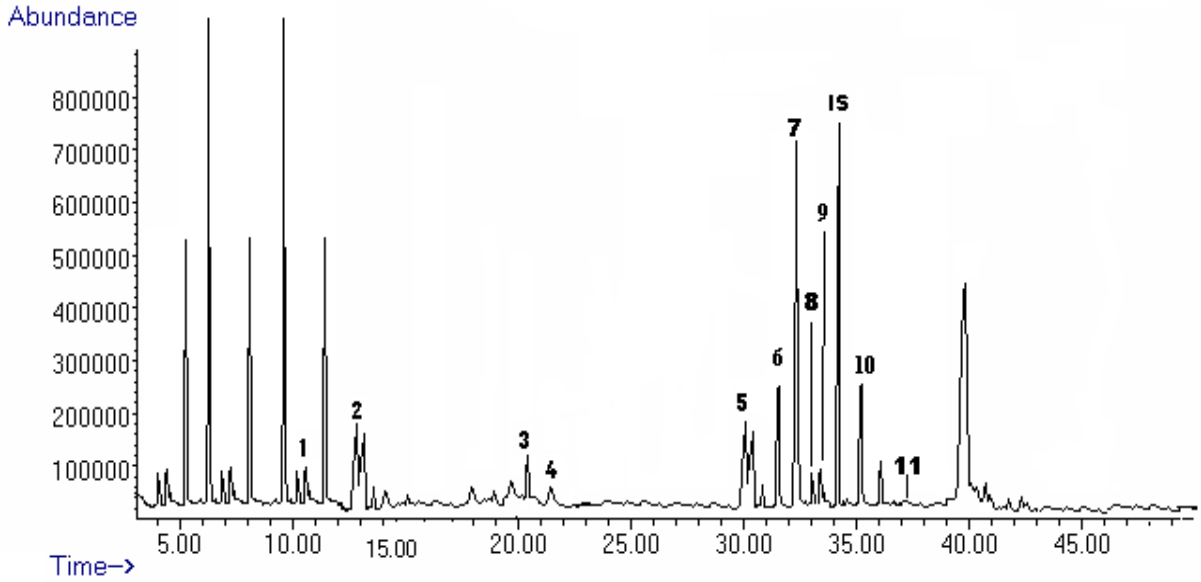

Table 5: HPLC results showing peak area and retention time for compounds detected in sample NS b 2.25% (w/w)

Peak No	**Sugars**	**Retention Time(RT)**	**% Quality**
1	Fructose	11.452	85.0
2	Arabinose	13.232	91.0
3	Maltose	22.120	90.0
4	Glucoronic acid	22.225	87.0
5	Mannose	30.121	90.0
6	Xylose	31.554	92.0
7	Glucose	32.261	98.0
8	Cellobiose	33.631	92.0
9	D-galactose	33.962	91.0
10	Sucrose	34.002	89.0
IS	Internal Standard	35.113	98.0
11	Xylobiose	37.247	68.0

Figure 6: HPLC chromatogram of sample NS a 2.25% (w/w) showing separation of sugars

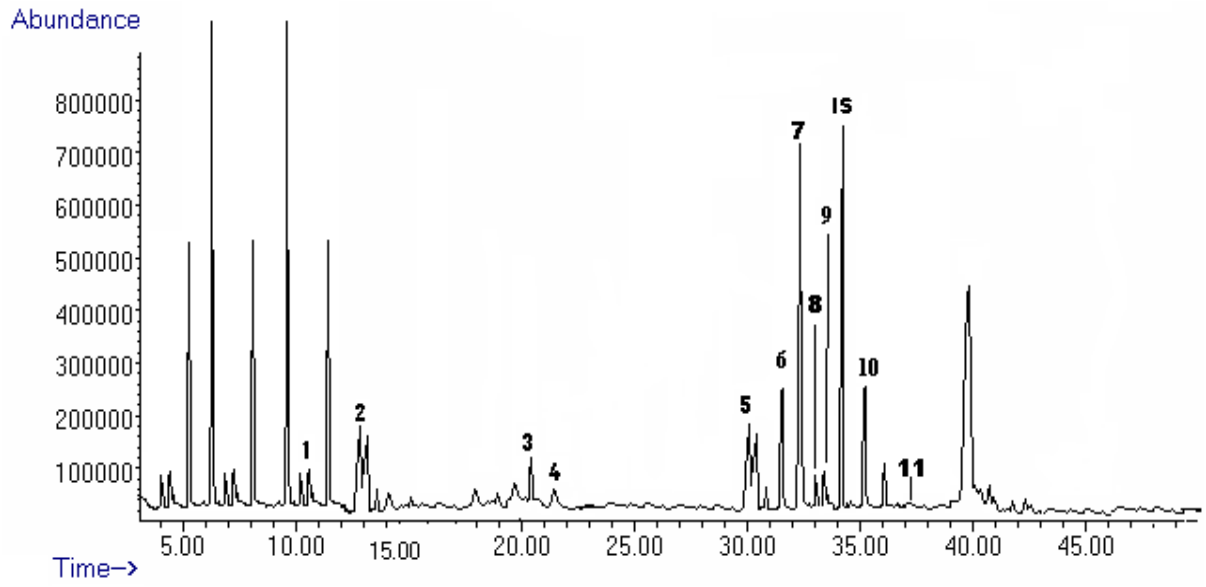

Table 6: HPLC results showing peak area and retention time for compounds detected in sample NS a 2.25% (w/w)

Peak No	**Sugars**	**Retention Time (RT)**	**% Quality**
1	Fructose	11.452	85.0
2	Arabinose	13.232	91.0
3	Maltose	22.120	90.0
4	Glucoronic acid	22.225	87.0
5	Mannose	30.121	90.0
6	Xylose	31.554	92.0
7	Glucose	32.261	98.0
8	Cellobiose	33.631	92.0
9	D-galactose	33.962	91.0
10	Sucrose	34.002	89.0
IS	Internal Standard	35.113	98.0
11	Xylobiose	37.247	68.0

Figure 7: HPLC chromatogram of sample SP17 a 1.2% (w/w) showing separation of sugars

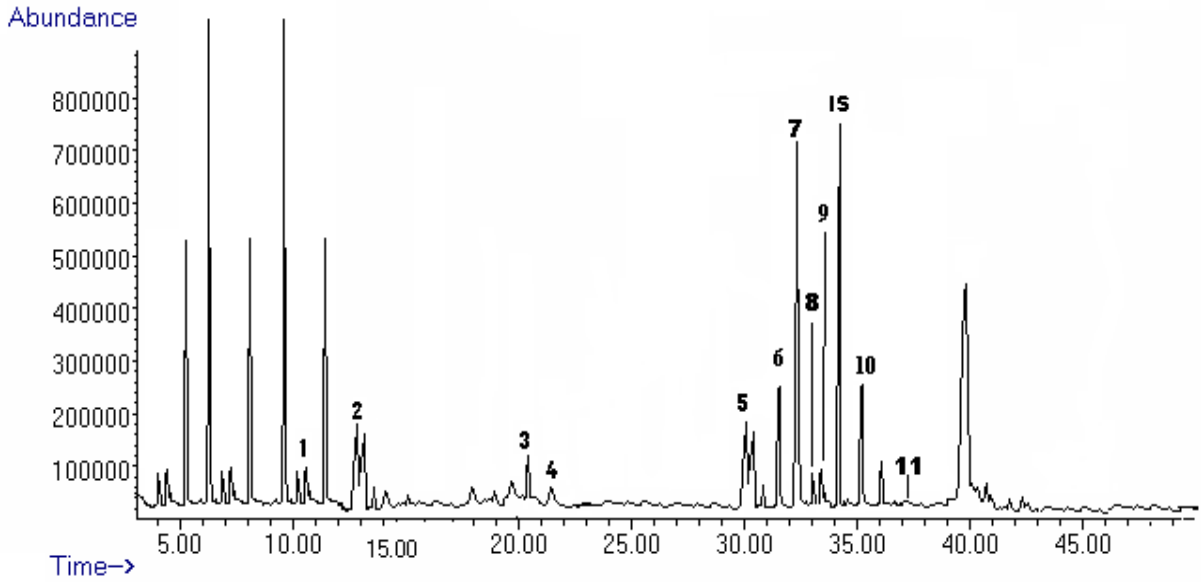

Table 7: HPLC results showing peak area and retention time for compounds detected in sample SP17 a 1.2% (w/w)

Peak No	**Sugars**	**Retention Time (RT)**	**% Quality**
1	Fructose	11.452	86.0
2	Arabinose	13.232	92.0
3	Maltose	22.120	91.0
4	Glucoronic acid	22.225	87.0
5	Mannose	30.121	90.0
6	Xylose	31.554	92.0
7	Glucose	32.261	94.0
8	Cellobiose	33.631	93.0
9	D-galactose	33.962	90.0
10	Sucrose	34.002	91.0
IS	Internal Standard	35.113	98.0
11	Xylobiose	37.247	67.0

Figure 8: HPLC chromatogram of sample SP17 b 1.2% (w/w) showing separation of sugars

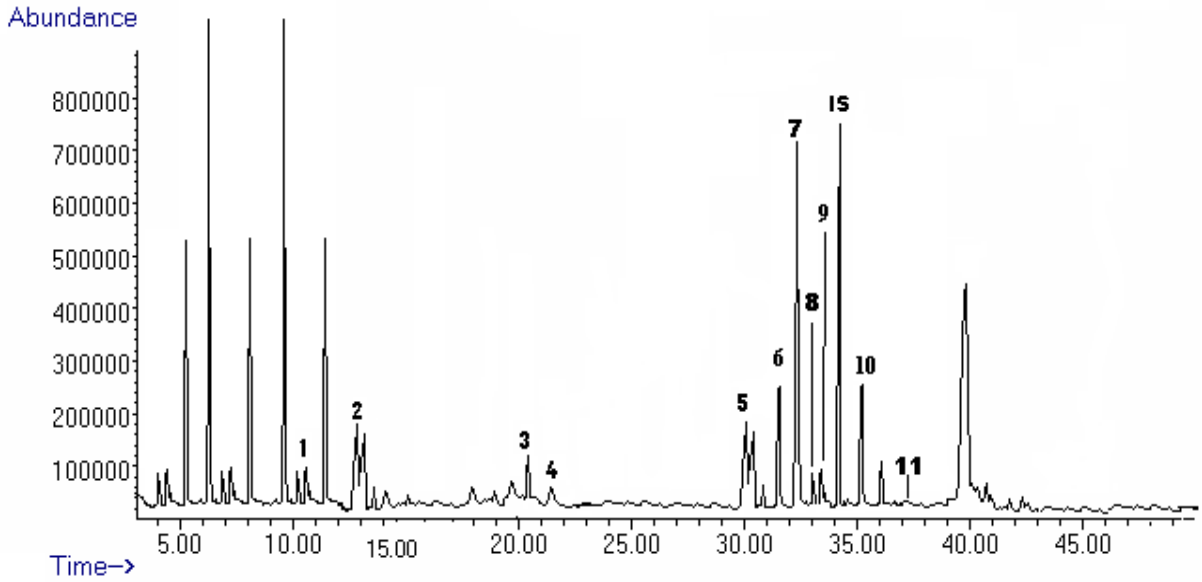

Table 8: HPLC results showing peak area and retention time for compounds detected in sample SP17 b 1.2% (w/w)

Peak No	**Sugars**	**Retention Time(RT)**	**% Quality**
1	Fructose	11.884	86.0
2	Arabinose	13.232	93.0
3	Maltose	22.060	90.0
4	Glucoronic acid	22.114	87.0
5	Mannose	30.008	90.0
6	Xylose	31.526	93.0
7	Glucose	32.247	96.0
8	Cellobiose	33.629	92.0
9	D-galactose	33.897	90.0
10	Sucrose	34.014	90.0
IS	Internal Standard	35.121	98.0
11	Xylobiose	37.198	66.0

Figure 9: HPLC chromatogram of sample SP08 b 1.2% (w/w) showing separation of sugars

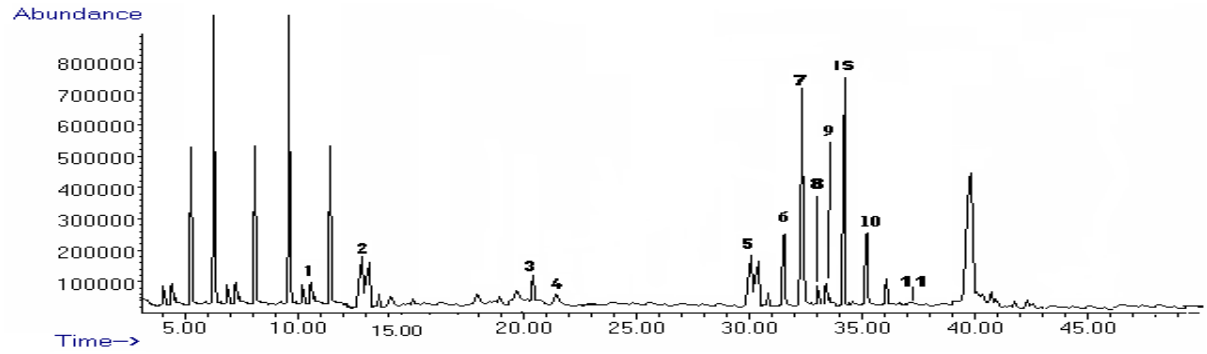

Table 9: HPLC results showing peak area and retention time for compounds detected in sample SP08 b 1.2% (w/w)

Peak No		**Sugars**	**Retention Time(RT)**	**% Quality**
1		Fructose	11.541	86.0
2		Arabinose	13.224	92.0
3		Maltose	22.059	90.0
4		Glucoronic acid	22.214	86.0
5		Mannose	30.008	90.0
6		Xylose	31.531	93.0
7		Glucose	32.245	98.0
8		Cellobiose	33.628	92.0
9		D-galactose	33.978	90.0
10		Sucrose	34.007	89.0
IS		Internal Standard	35.115	98.0
11		Xylobiose	37.254	67.0

Figure 10: HPLC chromatogram of sample BH a 2.25% (w/w) showing separation of sugars

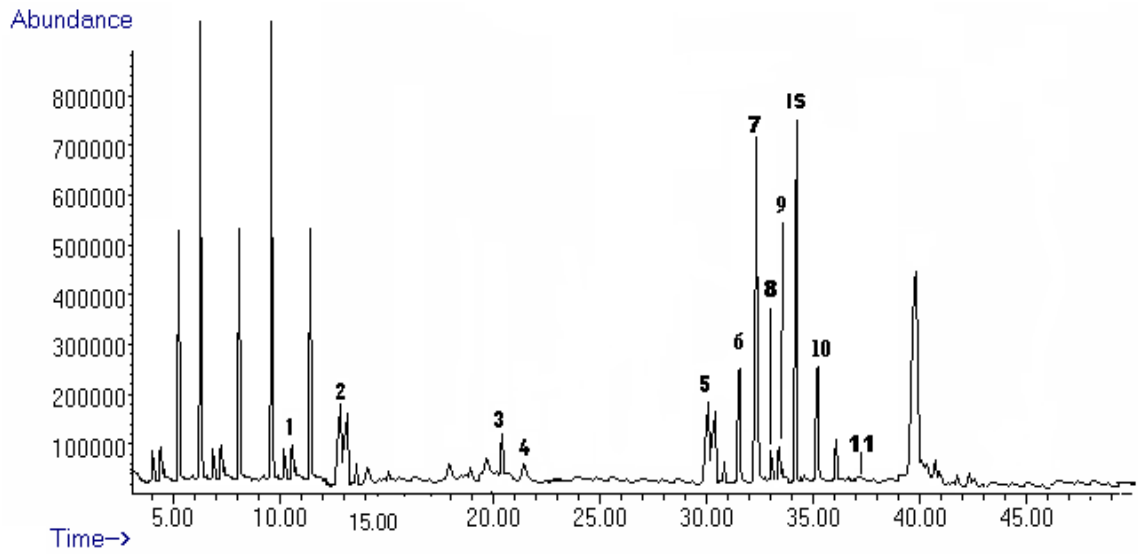

Table 10: HPLC results showing peak area and retention time for compounds detected in sample BH a 2.25% (w/w)

Peak No	**Sugars**	**Retention Time(RT)**	**% Quality**
1	Fructose	11.776	86.0
2	Arabinose	13.258	91.0
3	Maltose	22.061	90.0
4	Glucoronic acid	22.102	86.0
5	Mannose	30.004	90.0
6	Xylose	31.527	93.0
7	Glucose	32.243	98.0
8	Cellobiose	33.821	92.0
9	D-galactose	33.984	91.0
10	Sucrose	34.007	89.0
IS	Internal Standard	35.115	98.0
11	Xylobiose	37.211	66.0

Figure 11: HPLC chromatogram of sample BH b2.25% (w/w) showing separation of sugars

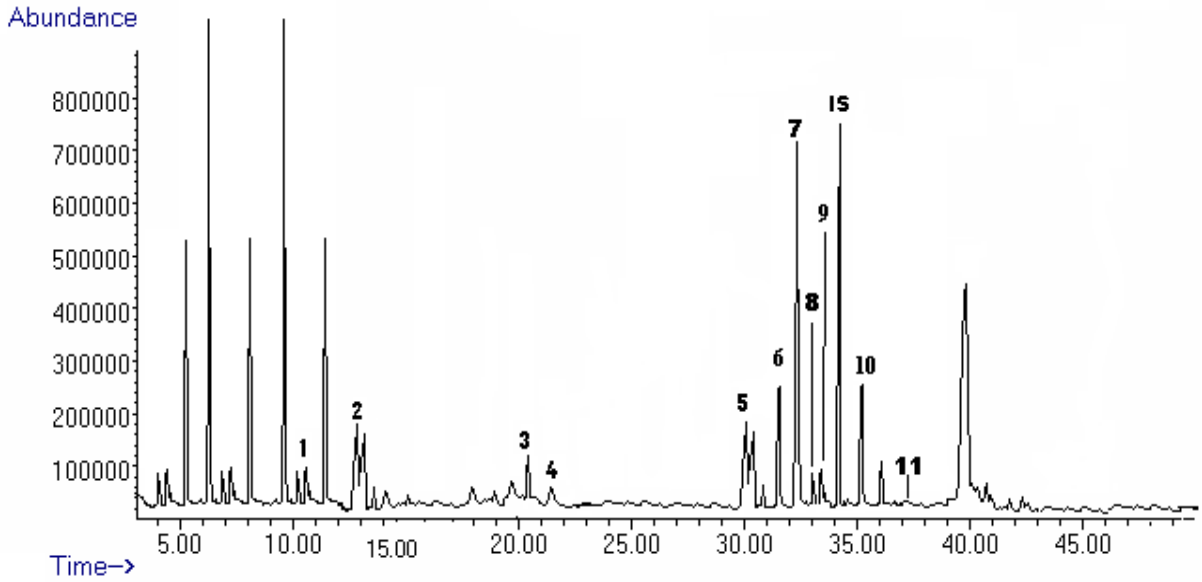

Table 11: HPLC results showing peak area and retention time for compounds detected in sample BH b 2.25% (w/w)

Peak No	**Sugars**	**Retention Time(RT)**	**% Quality**
1	Fructose	11.776	86.0
2	Arabinose	13.258	91.0
3	Maltose	22.061	90.0
4	Glucoronic acid	22.102	86.0
5	Mannose	30.004	90.0
6	Xylose	31.527	93.0
7	Glucose	32.243	98.0
8	Cellobiose	33.821	92.0
9	D-galactose	33.984	91.0
10	Sucrose	34.007	89.0
IS	Internal Standard	35.115	98.0
11	Xylobiose	37.211	66.0

Figure 12: HPLC chromatogram of sample NS a 1.2% (w/w) showing separation of sugars

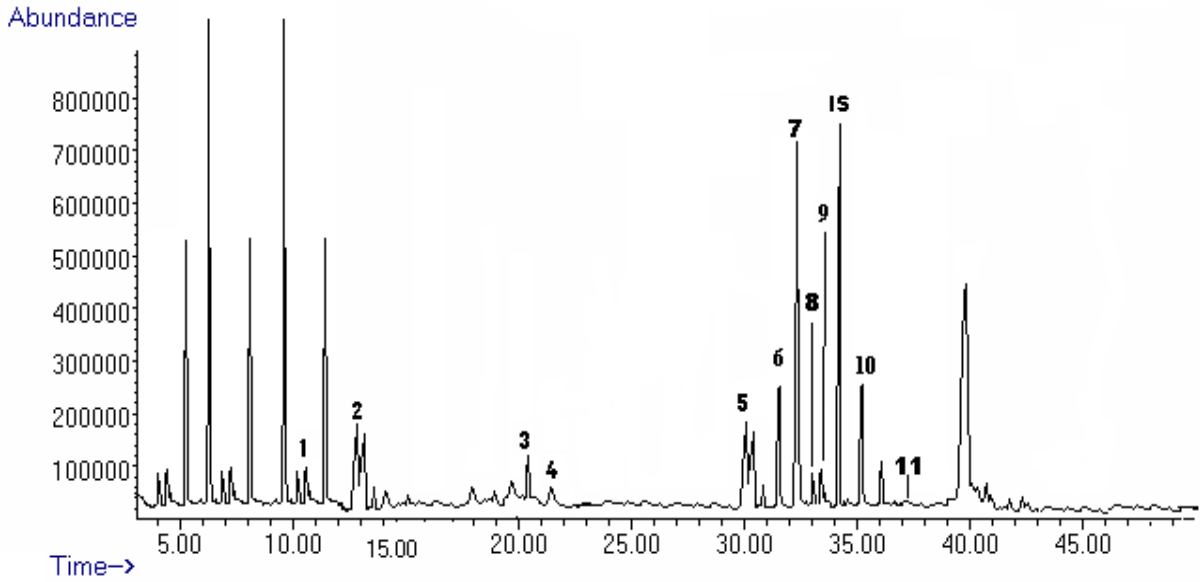

Table 12: HPLC results showing peak area and retention time for compounds detected in sample NS a 1.2% (w/w)

Peak No	**Sugars**	**Retention Time(RT)**	**% Quality**
1	Fructose	11.876	92.0
2	Arabinose	13.258	94.0
3	Maltose	22.059	91.0
4	Glucoronic acid	22.102	89.0
5	Mannose	30.008	92.0
6	Xylose	31.526	93.0
7	Glucose	32.245	99.0
8	Cellobiose	33.628	93.0
9	D-galactose	33.987	90.0
10	Sucrose	34.007	98.0
IS	Internal Standard	35.115	94.0
11	Xylobiose	37.214	74.0

Figure 13: HPLC chromatogram of sample BS a 1.2% (w/w) showing separation of sugars

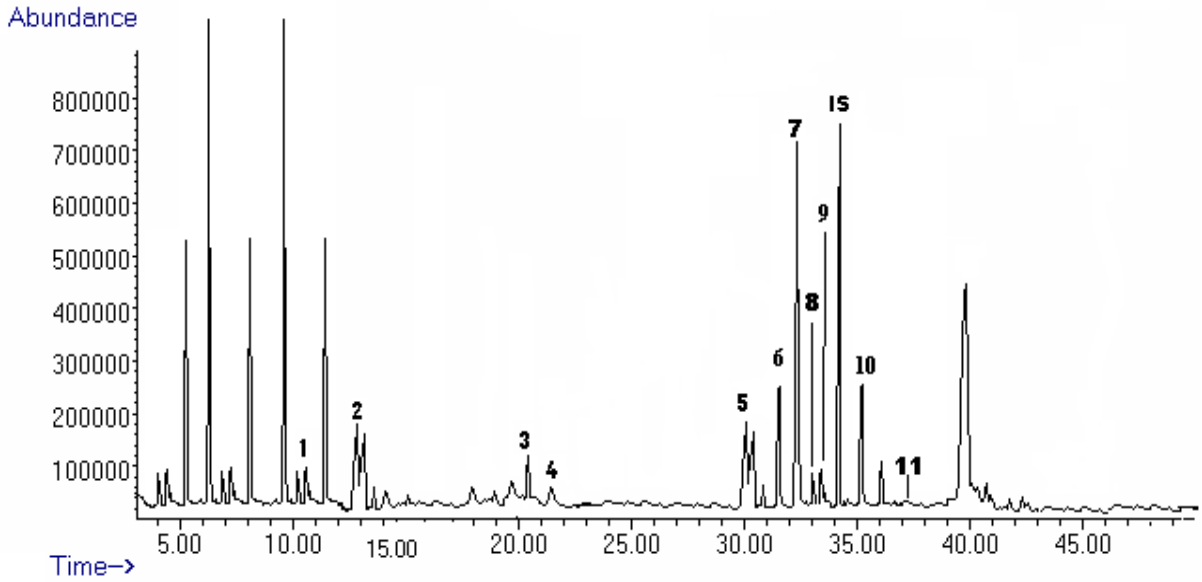

Table 13: HPLC results showing peak area and retention time for compounds detected in sample BS a 1.2% (w/w)

Peak No	**Sugars**	**Retention Time(RT)**	**% Quality**
1	Fructose	11.452	86.0
2	Arabinose	13.232	92.0
3	Maltose	22.120	91.0
4	Glucoronic acid	22.225	87.0
5	Mannose	30.121	90.0
6	Xylose	31.554	92.0
7	Glucose	32.261	94.0
8	Cellobiose	33.631	93.0
9	D-galactose	33.962	90.0
10	Sucrose	34.002	91.0
IS	Internal Standard	35.113	98.0
11	Xylobiose	37.247	66.0

Figure 14: HPLC chromatogram of sample BS b 1.2% (w/w) showing separation of sugars

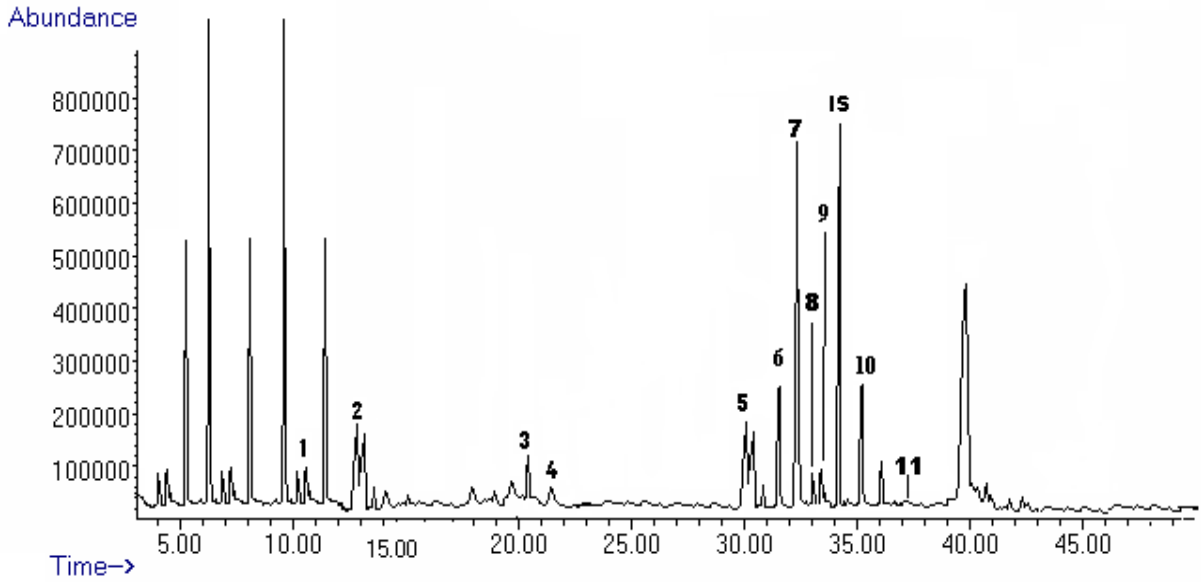

Table 14: HPLC results showing peak area and retention time for compounds detected in sample BS b 1.2% (w/w)

Peak No	**Sugars**	**Retention Time(RT)**	**% Quality**
1	Fructose	11.452	86.0
2	Arabinose	13.232	92.0
3	Maltose	22.120	91.0
4	Glucoronic acid	22.225	87.0
5	Mannose	30.121	90.0
6	Xylose	31.554	92.0
7	Glucose	32.261	94.0
8	Cellobiose	33.631	93.0
9	D-galactose	33.962	90.0
10	Sucrose	34.002	91.0
IS	Internal Standard	35.113	98.0
11	Xylobiose	37.247	66.0

Figure 15: HPLC chromatogram of sample NH b 2.25% (w/w) showing separation of sugars

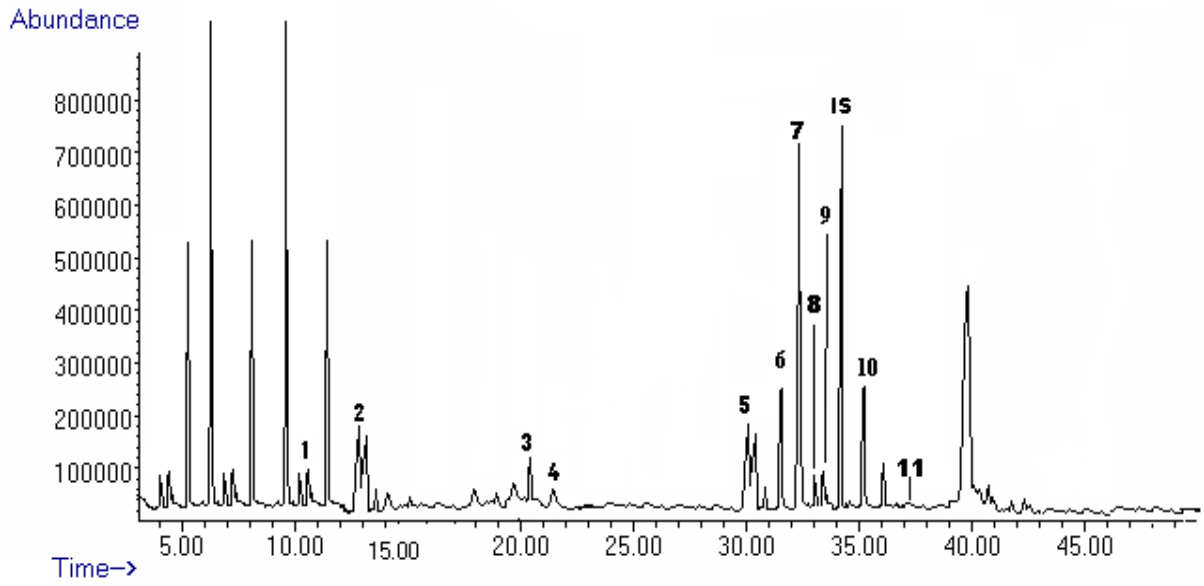

Table 15: HPLC results showing peak area and retention time for compounds detected in sample NH b 2.25% (w/w)

Peak No	**Sugars**	**Retention Time (RT)**	**% Quality**
1	Fructose	11.452	85.0
2	Arabinose	13.232	91.0
3	Maltose	22.120	90.0
4	Glucoronic acid	22.225	87.0
5	Mannose	30.121	90.0
6	Xylose	31.554	92.0
7	Glucose	32.261	98.0
8	Cellobiose	33.631	92.0
9	D-galactose	33.962	91.0
10	Sucrose	34.002	89.0
IS	Internal Standard	35.113	98.0
11	Xylobiose	37.247	67.0

Figure 16: HPLC chromatogram of sample NH b 1.2% (w/w) showing separation of sugars

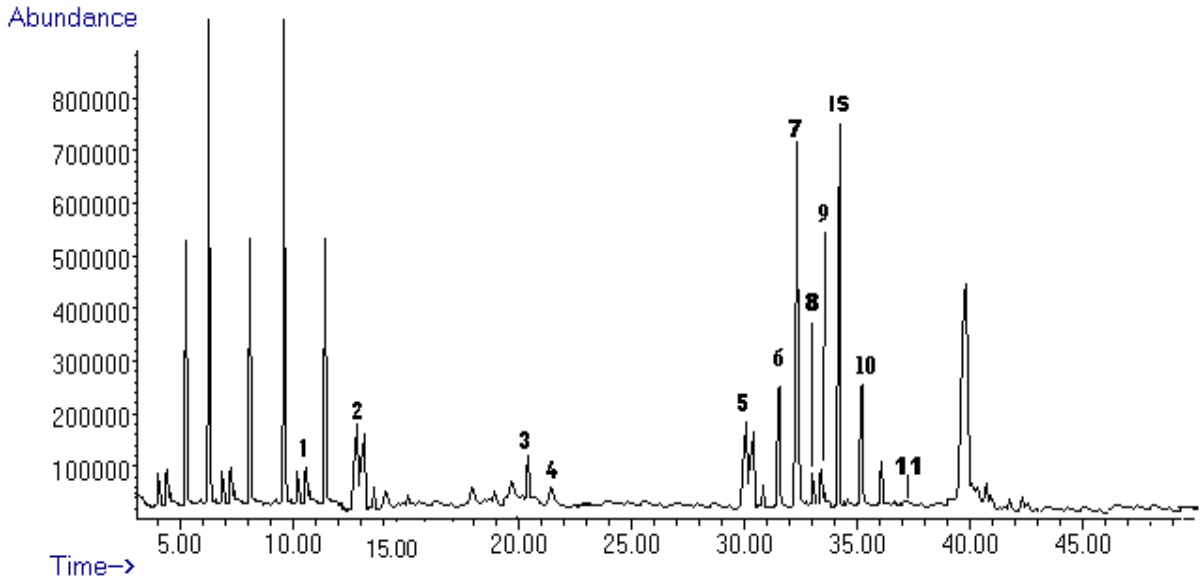

Table 16: HPLC results showing peak area and retention time for compounds detected in sample NH b 1.2 % (w/w)

Peak No	**Sugars**	**Retention Time (RT)**	**% Quality**
1	Fructose	11.452	86.0
2	Arabinose	13.232	92.0
3	Maltose	22.120	91.0
4	Glucoronic acid	22.225	87.0
5	Mannose	30.121	90.0
6	Xylose	31.554	92.0
7	Glucose	32.261	94.0
8	Cellobiose	33.631	93.0
9	D-galactose	33.962	90.0
10	Sucrose	34.002	91.0
IS	Internal Standard	35.113	98.0
11	Xylobiose	37.247	66.0

Figure 17: HPLC chromatogram of sample BS a 2.25% (w/w) showing separation of sugars

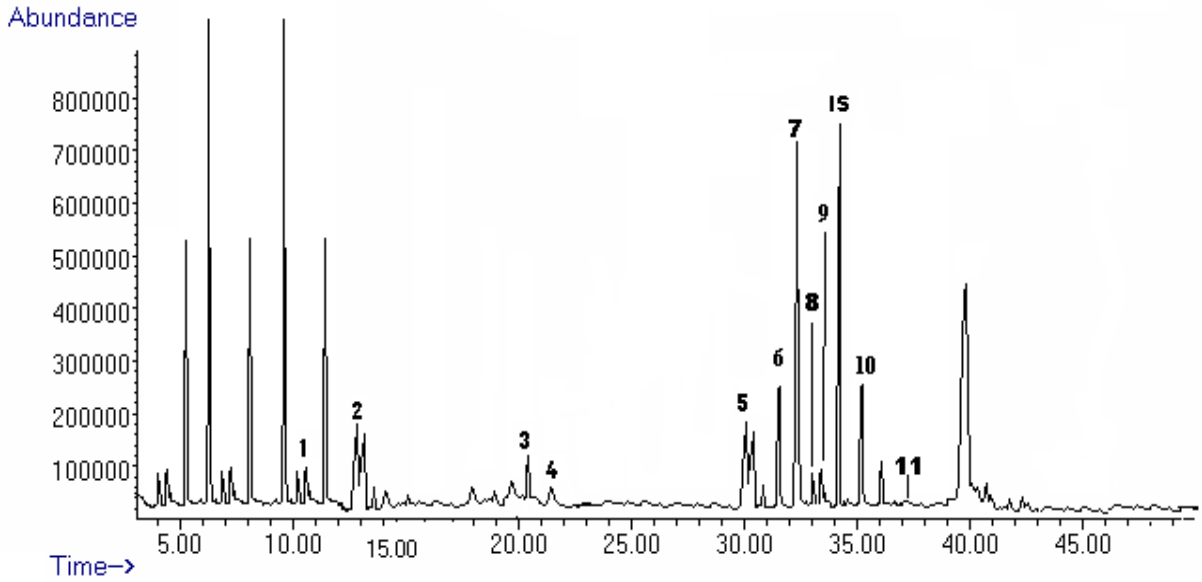

Table 17: HPLC results showing peak area and retention time for compounds detected in sample BS a 2.25 % (w/w)

Peak No	**Sugars**	**Retention Time(RT)**	**% Quality**
1	Fructose	11.884	85.0
2	Arabinose	13.232	92.0
3	Maltose	22.060	90.0
4	Glucoronic acid	22.114	87.0
5	Mannose	30.008	90.0
6	Xylose	31.526	93.0
7	Glucose	32.247	97.0
8	Cellobiose	33.629	93.0
9	D-galactose	33.897	90.0
10	Sucrose	34.014	91.0
IS	Internal Standard	35.121	98.0
11	Xylobiose	37.198	66.0

Figure 18: HPLC chromatogram of sample BS b 2.25% (w/w) showing separation of sugars

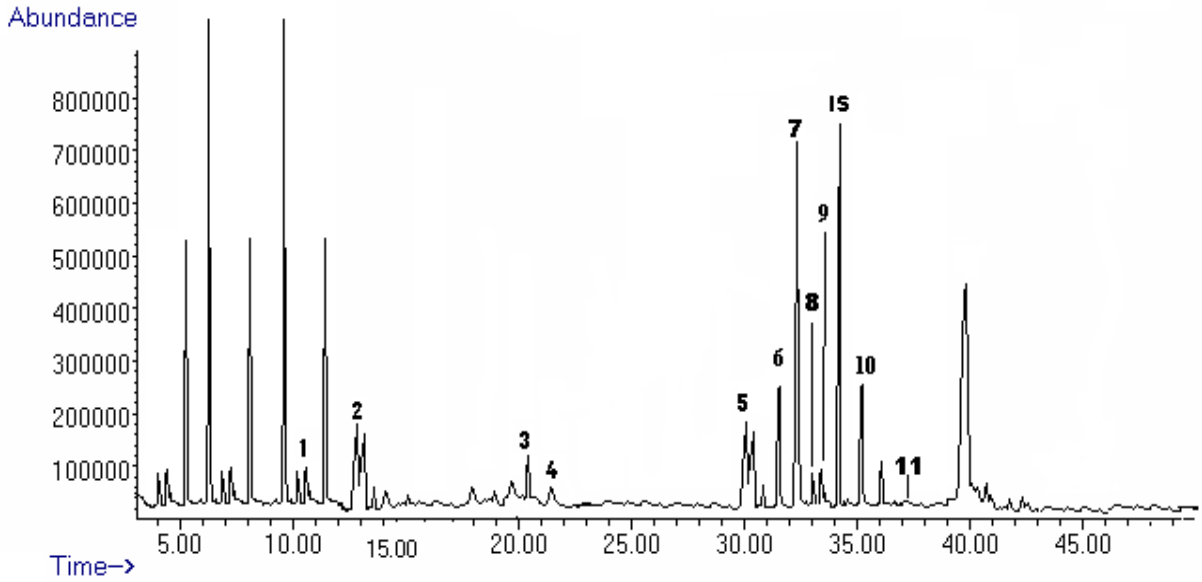

Table 18: HPLC results showing peak area and retention time for compounds detected in sample BS b 2.25 % (w/w)

Peak No	**Sugars**	**Retention Time(RT)**	**% Quality**
1	Fructose	11.541	86.0
2	Arabinose	13.224	92.0
3	Maltose	22.059	90.0
4	Glucoronic acid	22.214	86.0
5	Mannose	30.008	90.0
6	Xylose	31.531	93.0
7	Glucose	32.245	98.0
8	Cellobiose	33.628	92.0
9	D-galactose	33.978	90.0
10	Sucrose	34.007	89.0
IS	Internal Standard	35.115	98.0
11	Xylobiose	37.254	67.0

Figure 19: HPLC chromatogram of sample SP08 b 2.25% (w/w) showing separation of sugars

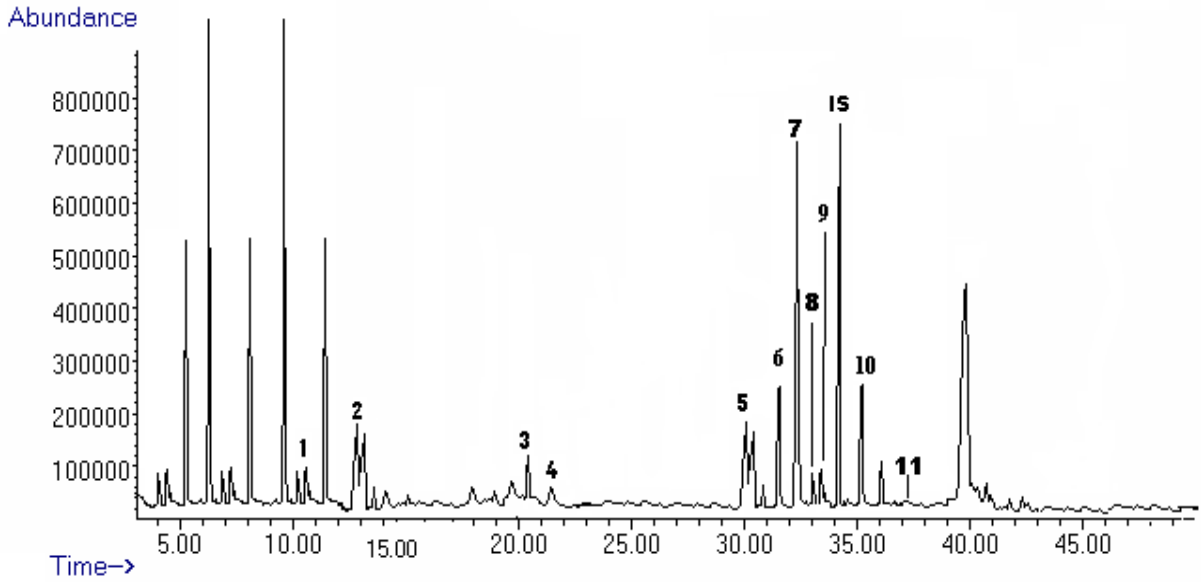

Table 19: HPLC results showing peak area and retention time for compounds detected in sample SP08 b 2.25 % (w/w)

Peak No	**Sugars**	**Retention Time (RT)**	**% Quality**
1	Fructose	11.876	86.0
2	Arabinose	13.258	92.0
3	Maltose	22.059	90.0
4	Glucoronic acid	22.102	86.0
5	Mannose	30.008	90.0
6	Xylose	31.526	93.0
7	Glucose	32.245	98.0
8	Cellobiose	33.628	92.0
9	D-galactose	33.987	90.0
10	Sucrose	34.007	89.0
IS	Internal Standard	35.115	98.0
11	Xylobiose	37.214	67.0